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DISSERTATION

Genotyping of the polymorphic drug metabolizing enzymes cytochrome P450 2D6 and 1A1, and N-acetyltransferase 2 in a Russian sample

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Abstrakt

Die Umwandlung in wasserlösliche Verbindungen, die renal ausgeschieden werden können, ist ein grundlegendes Prinzip im Abbau von Fremdstoffen. Hierbei unterscheidet man Phase-I- und Phase-II-Reaktionen. Die Aktivität vieler Phase-I- und Phase-II-Enzyme ist genetisch beeinflusst und kann starke interindividuelle Unterschiede im Metabolismus von Fremdstoffen verursachen und dadurch das Krebsrisiko und das Risiko für Arzneimittelnebenwirkungen beeinflussen. Die Häufigkeitsverteilungen der Allele der Gene, die Phase-I- und Phase-II-Enzyme kodieren, zeigen eine große interethnische Varianz. Die Polymorphismen dieser Enzyme wurden bisher jedoch noch nicht in der größten slawischen Volksgruppe, der russischen, untersucht. An der vorliegenden Studie nahm eine Gruppe von 325 Personen russischer Abstammung teil - gesunde Probanden bzw. Patienten, die nicht an einer malignen Erkrankung litten. Die Polymorphismen von zwei Enzymen der Phase I, CYP1A1 und CYP2D6, und von einem Enzym der Phase II, NAT2, wurden mittels PCR-RFLP-Genotypisierung und Real-time-PCR-Verfahren komplett untersucht. Die Häufigkeit der *CYP1A1* Allele mit hoher Aktivität, *CYP1A1*2A* und *CYP1A1*2B*, betrug 4,6% (3,1%-6,5%) bzw. 5,1% (3,5%-7,1%). Die Häufigkeiten der genetischen Varianten von *CYP1A1* waren: m1 (3801T>C) - 9,8% (95% Vertrauensbereich, 7,7%-12,4%), m2 (2455A>G) - 5,0% (95% VB, 3,5%-7,1%), m4 (2453C>A) - 2,5% (1,4%-4,0%), m5 (-4335G>A) - 25,8% (22,5%-29,4%), m6 (-3219C>T) - 6,0% (4,3%-8,1%), und m7 (-3229G>A) - 2,9% (1,8%-4,5%). Die Mutation m3, die bisher nur bei Afrikaner gefunden wurde, konnten wir nicht nachweisen. 5,9% (3,5%-9,2%) aller Probanden waren CYP2D6 Langsam-Metabolisierer und 3,4% (1,7%-6,3%) wurden als Ultraschnell-Metabolisierer identifiziert (*CYP2D6*1x1/*1*). Bei der Genotypisierung von acht verschiedenen Punktmutationen im *NAT2*-Gen ergab sich für 59,7% (54,1%-65,1%) der Studienteilnehmer ein Genotyp, der mit einer Langsam-Acetylierer-Status einhergeht. 34,7% (29,6%-40,2%) der Probanden hatten ein und 5,6% (3,3%-8,6%) zwei für die Schnellacetylierung kodierende Allele. Die Allelverteilung der für die wichtigsten Enzyme im Arzneimittelstoffwechsel kodierenden Gene ist bei Russen ähnlich wie bei anderen Kaukasiern. Es kann deshalb erwartet werden, dass die genetisch-bedingten Unterschiede in der Wirksamkeit und im Auftreten von Arzneimittelnebenwirkungen in der russischen Bevölkerung vergleichbar sind mit denen in anderen europäischen Populationen.

Schlagworte: Cytochrom P450, CYP1A1, CYP2D6, NAT2, Russische Bevölkerung, Genotyp

Abstract

The basic principle of drug and xenobiotic metabolism in the body is to make them more water soluble and thus more readily excreted in the urine. Genetic polymorphisms of phases I and II xenobiotic transformation reactions are known to contribute considerably to interindividual variations in the metabolism of numerous drugs and xenobiotics and to associate with altered risk of adverse drug reactions and some cancers. The frequency of functionally important mutations and alleles of genes coding for xenobiotic metabolizing enzymes shows a wide ethnic variation. However, little is known of the frequency distribution of the major allelic variants in the Russian population. In this study we investigated 325 individuals of Russian origin, who were healthy volunteers or patients without malignant diseases. Our study included the complete investigation of two enzymes of phase I, CYP1A1 and CYP2D6, and one phase II enzyme, NAT2, using PCR-RFLP genotyping and LightCycler method. The frequencies of the *CYP1A1* high-activity alleles, *CYP1A1*2A* and *CYP1A1*2B*, were 4.6% (3.1%-6.5%) and 5.1% (3.5%-7.1%), respectively. The mutations m1 (3801T>C), m2 (2455A>G), m4 (2453C>A), m5 (-4335G>A), m6 (-3219C>T), and m7 (-3229G>A) of *CYP1A1* occurred in 9.8% (95% confidence interval, 7.7%-12.4%), 5.0% (95% C. I., 3.5%-7.1%), 2.5% (1.4%-4.0%), 25.8% (22.5%-29.4%), 6.0% (4.3%-8.1%), and 2.9% (1.8%-4.5%) of alleles, respectively. We did not find the m3 mutation, which has only been detected in Africans up to now. 5.9% (3.5%-9.2%) of all subjects were CYP2D6 poor metabolizers, whereas 3.4% (1.7%-6.3%) were identified as ultra-rapid metabolizers (*CYP2D6*1x1/*1*). Genotyping eight different single nucleotide polymorphisms in the *NAT2* gene provided a genotype associated with slow acetylation in 59.7% (54.1%-65.1%) of individuals, 34.7% (29.6%-40.2%) of participants carried at least one allele encoding rapid acetylation, and 5.6% (3.3%-8.6%) were homozygous for the rapid-acetylation allele (wild-type allele **4* or mutant allele **12A*). The overview of allele distribution of the important drug and xenobiotic metabolizing enzymes among Russians shows that the allele frequency is similar to that of other Caucasians. Therefore it may be expected that drug side effects and efficacy problems due to an individual's genetic background are similar compared to those in other European populations.

Keywords: Cytochrome P450, CYP1A1, CYP2D6, NAT2, Russians, Genotype

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Abbreviations

bp	basepairs
95% C.I.	95% confidence interval
CYP1A1	cytochrome P450 1A1 (enzyme)
CYP1A1	cytochrome P450 1A1 (gene)
CYP2D6	cytochrome P450 2D6 (enzyme)
CYP2D6	cytochrome P450 2D6 (gene)
BSA	bovine serum albumin
dNTP	deoxyribonucleotide triphosphate (dATP, dCTC, dGTP and dTTP)
DME	drug-metabolizing enzyme
DMSO	dimethylsulfoxide
EM	extensive metabolizer
IM	intermediate metabolizer
NAT2	arylamine *N*-acetyltransferase 2 (enzyme)
NAT2	arylamine *N*-acetyltransferase 2 (gene)
nt	nucleotide
PAH	polycyclic aromatic hydrocarbon
PCR	polymerase chain reaction
PM	poor metabolizer
RFLP	restriction fragment length polymorphism
rpm	rounds per minute
Taq	Thermus aquaticus
U	unit
UM	ultrarapid metabolizer
wt	wild type

1 Introduction

All organisms are exposed constantly and unavoidably to foreign chemicals, or xenobiotics, which include both man-made and natural chemicals such as drugs, industrial chemicals, pesticides, pollutants, pyrolysis products in cooked food, alkaloids, secondary plant metabolites, and toxins produced by molds, plants and animals. The physical property that enables many xenobiotics to be absorbed through the skin, lungs, or gastrointestinal tract, namely their lipophilicity, is an obstacle to their elimination because lipophilic compounds can be readily reabsorbed. Consequently, the elimination of xenobiotics often depends on their conversion to water-soluble compounds by a process known as biotransformation, which is catalyzed by enzymes in the liver and other tissues.

The activity of these enzymes varies broadly between individuals from absence to high activity and this variance can be responsible for adverse or toxic effects of drugs and xenobiotics or plays a key role in the etiopathology of several malignancies. Their enzymatic activities depend on hereditary polymorphisms of the genes which encode these enzymes. The frequency of functionally important mutations and alleles has been described in different populations revealing a broad ethnical variation. It is this aspect of ethnicity of foreign compound metabolism that the present work deals with taking the Russian population as an example. Genetic variability of clinically important biotransformation steps is investigated and compared to published data reflecting variability in other ethnic groups. It is expected that the knowledge of these variabilities in a specific population will improve drug treatment: firstly, by individualizing of drug dose according to the respective genetic trait of a patient; secondly, by reducing the incidence of side effects. Moreover, disease susceptibility has been shown to partly depend on the genetic make up of the enzymes which are involved in xenobiotic metabolism. For a better understanding of the results, the following chapters will explain the basic principles of the cytochrome P450 enzyme system and of the arylamine *N*-acetyltransferase, the main targets of the present work.

An important consequence of biotransformation is that the physical properties of a xenobiotic are generally changed from those favoring absorption (lipophilicity) to those favoring excretion (hydrophilicity). A change in pharmacokinetic behavior is not the only result of xenobiotic biotransformation, but in some cases it is the most important one. Xenobiotics exert a variety of effects on biological systems. These may be beneficial – as in the case of drugs, or deleterious – as in the case of poisons. These effects are dependent on the physicochemical properties of the xenobiotic and often altered by biotransformation. Thus some drugs must undergo

biotransformation to be efficacious (because it is the metabolite of the drug, and not the drug itself, that exerts the pharmacological effect). Similarly, many xenobiotics must undergo biotransformation to exert their characteristic toxic or tumorogenic effects. Many chemicals would be considerably less toxic or tumorogenic if they were not converted to reactive metabolites by xenobiotic-biotransformation enzymes. In most cases, however, biotransformation terminates the effectiveness of a drug and lessens the toxicity of xenobiotics. Thus, enzymes catalyzing biotransformation reactions often determine the intensity and duration of the action of drugs and play a key role in chemical toxicity and chemical tumor genesis.

The reactions catalyzed by xenobiotic-biotransforming enzymes are generally divided into two groups, called phase I and phase II (Table 1). Phase I reactions involve oxidation, hydrolysis and reduction. These reactions expose or introduce a functional group (-OH, -NH_2, -SH or -COOH), and usually result in only a small increase in hydrophilicity. Phase II **biotransformation** reactions include glucuronidation, sulfation, acetylation, methylation, conjugation with glutathione (mercaptopuric acid synthesis) and conjugation with amino acids (such as glycine, taurine, and glutamic acid) (Pau86).

Table 1: List of some drug metabolizing enzymes.

Phase I reactions		
Oxidation	Hydroxylation, *N*-oxidation, *S*-oxidation, *N*-dealkylation, *O*-dealkylation, desamination, desulfation, oxidative dehalogenation	Cytochrome P450-monooxigenases
	Dehydration	Alcohol dehydrogenase
	dehydration of amines	Monoaminoxidases
	N-oxidation, *S*-oxidation	Flavin monooxygenases
Reduction	Dehalogenisation of nitrogroups	Cytochrome P450-monooxigenases
Hydrolysis	Hydrolysis of epoxides	Epoxide hydrolases
Others	Oxidation of radicals	Superoxide dismutases
	peroxidation	glutathione peroxidases
Phase II reactions		
Conjugation	Glucosylation	UDP-glucuronosyltransferase
	sulfation	sulfotransferases
	acetylation	*O*-, *N*-acetyltransferases
	methylation	*O*-, *N*-, *S*-methyltransferases
	glutathione *S*-conjugation	glutathione *S*-transferases

In these reactions, specific cofactors react with functional groups that are either present in xenobiotics or are introduced/exposed during phase I biotransformation. Most phase II biotransformation reactions result in a large increase in the hydrophilicity of the xenobiotic, hence they greatly promote the excretion of foreign chemicals. Phase II biotransformation of xenobiotics may or may not be preceded by phase I biotransformation. For example, morphine, heroine, and codeine are all converted to morphine-3-glucuronide. In the case of morphine, this metabolite is formed by direct conjugation with glucuronic acid. In the other two cases, however, conjugation with glucuronic acid is preceded by phase I biotransformation: hydrolysis (deacetylation) in the case of heroine, and *O*-demethylation (involving oxidation by cytochrome P450) in the case of codeine. In general, phase II biotransformation does not precede phase I biotransformation, although there are exceptions to this rule. For example, some sulfate steroids (including some steroid disulfates) are hydroxylated by cytochrome P450.

Xenobiotic-biotransforming enzymes are widely distributed throughout the body, and are present in several subcellular compartments. The liver is the organ with the highest concentration of enzymes catalyzing biotransformation reactions. These enzymes are also located in the skin, lungs, nasal mucosa, eyes, and gastrointestinal tract. In the liver and in most other organs, they are located primarily in the endoplasmatic reticulum (microsomes) or in the soluble fraction of the cytoplasm (cytosol), with a smaller concentration in the mitochondria, nuclei, and lysosomes. By extracting and biotransforming xenobiotics absorbed from the gastrointestinal tract, the liver limits the systemic bioavailability of orally ingested xenobiotics, a process known as first pass elimination. In some cases, xenobiotic biotransformation in the intestine contributes significantly to the first pass elimination of foreign chemicals. For example, the oxidation of cyclosporin by cytochrome P450 and the conjugation of morphine with glucuronic acid in the small intestine limit the systemic bioavailability of these drugs. Under certain circumstances, the oxidation of ethanol to acetaldehyde in the gastric mucosa reduces the systemic bioavailability of alcohol. Some extrahepatic tissues contain high levels of xenobiotic-biotransforming enzymes, but their small size minimizes their overall contribution to the biotransformation of xenobiotics. For example, certain xenobiotic-biotransforming enzymes (such as cytochrome P450 enzymes, flavin-containing monooxygenases, glutathione *S*-transferases and carboxylesterases) are present in the nasal epithelium at levels that rival those found in the liver. The nasal epithelium plays an important role in the biotransformation of inhaled xenobiotics, including odorants, but it is quantitatively insignificant in the biotransformation of orally ingested xenobiotics (Bri93).

1.1 Cytochrome P450 enzyme system

Among the phase I biotransformation enzymes, the cytochrome P450 system ranks first in terms of catalytic versatility and the sheer number of xenobiotics it detoxifies or activates to reactive intermediates (Gue87, Wat91). The highest concentration of P450 enzymes involved in xenobiotic biotransformation is found in the endoplasmatic reticulum of the liver (microsomes), but P450 enzymes are present in almost all tissues. Microsomal P450 enzymes play a very important role in the regulation of the intensity and duration of drug action, in the detoxication of xenobiotics, and in the activation of xenobiotics to toxic and tumorogenic metabolites. In humans, about 40 different microsomal and mitochondrial P450 enzymes play key role by catalyzing reactions in:

- the metabolism of drugs, environmental pollutants and other xenobiotics;
- the biosynthesis of steroid hormones;
- the oxidation of unsaturated fatty acids to intracellular messengers;
- the stereo- and regio-specific metabolism of fat-soluble vitamins.

This underscores the catalytic flexibility of cytochrome P450.
All P450 enzymes are heme-proteins. The heme iron in cytochrome P450 is usually in the ferric (Fe^{3+}) state. When reduced to the ferrous (Fe^{2+}) state, cytochrome P450 can bind ligands such as O_2 and carbon monoxide (CO). The basis reaction catalyzed by cytochrome P450 is monooxygenation in which one atom of oxygen is incorporated into a substrate (RH); the other one is reduced to water with reducing equivalents derived from NADPH:

$$\text{Substrate (RH)} + O_2 + \text{NADPH} + H^+ \rightarrow \text{Product (ROH)} + NADP^+ + H_2O$$

Figure 1: P450-dependent oxygenation reaction.
R – organic chemical that is transformed in the catalyzed reaction

The liver microsomal P450 enzymes involved in xenobiotic biotransformation belong to three main P450 gene families, namely CYP1, CYP2 and CYP3. Liver microsomes also contain P450 enzymes encoded by the CYP4 gene family, the substrates of which include several fatty acids and eicosanoids but relatively few xenobiotics.
The level and activity of each P450 enzyme have been shown to vary from one individual to the next, due to environmental and genetic factors (Mey94, Shim94). Decreased P450 activity can result from (1) a genetic mutation that either blocks the synthesis of a P450 enzyme or leads to the synthesis of a catalytically compromised or inactive enzyme, (2) exposure to an

environmental factor (such as an infectious disease or a xenobiotic) that suppresses P450 enzyme expression, or (3) exposure to a xenobiotic that inhibits or inactivates a preexisting P450 enzyme. By inhibiting cytochrome P450, one drug can impair the biotransformation of another, which can lead to an excessive pharmacological or toxicological response to the second drug. In this regard, inhibition of cytochrome P450 mimics the effects of a genetic deficiency in P450 enzyme expression. Increased P450 enzyme activity can result from (1) gene duplication leading to overexpression of a P450 enzyme, (2) exposure to environmental factors, such as xenobiotics, that induce the synthesis of cytochrome P450, or (3) stimulation of a preexisting enzyme by a xenobiotic. By inducing cytochrome P450 one drug can stimulate the metabolism of a second drug and thereby decrease or increase its therapeutic effect. A dramatic effect of this type of drug interaction is the induction of ethinylestradiol metabolism by phenobarbital and rifampin, which can decrease the contraceptive effect of the former drug and lead to pregnancy. Allelic variants, which arise by point mutations in the wild-type gene, are another source of interindividual variation in P450 activity. Amino acid substitution can increase or, more commonly, decrease P450 enzyme activity, although the effect may be substrate-dependent. Examples of genetic factors that influence P450 activity are given below. The environmental factors which are known to affect P450 levels include medications (e.g. barbiturates, rifampin, isoniazid), food (e.g. cruciferous vegetables, charcoal-broiled beef), social habits (e.g. alcohol consumption, cigarette smoking), and disease status (diabetes, inflammation, hyperthyroidism and hypothyroidism). When environmental factors influence P450 enzyme levels, a considerable variation may be observed when xenobiotic biotransformation (e.g. drug metabolism) is measured repeatedly in the same individual. This variation is not observed when alterations in P450 activity are determined genetically.

Cytochrome P450 enzymes play a dual role in the organism. On the one hand, they inactivate the drug/xenobiotic and prepare it for excretion. On the other hand, they are also capable of activating foreign chemicals to highly reactive toxic intermediates that might act as carcinogens or mutagens (Table 2). Hence, the accumulation of null alleles may convey some as yet unspecified advantage to heterozygous carriers (having only 1 defective allele).

Table 2: The role of enzymes of phases I and II in the biotransformation of drugs, toxic substrates and carcinogens.

Toxic substrates, carcinogens	Drugs	Prodrugs	Potential carcinogens
↓	↓	↓	↓
detoxification	deactivation	activation	bioactivation
Phase I of biotransformation			
↓	↓	↓	↓
inactive metabolite		active metabolite	
↓	↓	↓	↓
loss of toxicity/ carcinogenesis	loss of pharmacodynamic effects	appearance	appearance of toxicity/ carcinogenesis
↓	↓	↓	↓
Further detoxification during the phase II of biotransformation			
↓	↓	↓	↓
Inactive water soluble or bile soluble metabolites			

1.1.1 Evolution of CYP450 genes

P450s are found throughout the animal and plant kingdoms. If the phylogenetic tree is examined and correlated with catalytic activities of P450s, several suggestions can be made concerning P450 evolution. The earliest P450s are those that now metabolize steroids and fatty acids. The fatty acid-metabolizing P450IV family and the steroid-inducible P450III genes diverged more than 1 billion years ago. The P450I and P450II gene families formed about 800 million years ago and these genes are now responsible for the metabolism of drugs and carcinogens. Finally, about 400 to 600 million years ago, the P450II gene family expanded into eight gene subfamilies. It has been suggested that this increase in the number of P450 genes was related to the emergence of mammals onto land several million years after plants had been established (Nels87). The presents of detoxifying enzymes allowed animals to survive in this hostile environment.

1.1.2 Cytochrome P450 1A1 (CYP1A1)

A phenotypic polymorphism affecting CYP1A1 activity in humans was first described in 1973 by Kellermann *et al*. (Kel73). The trimodal pattern of polycyclic aromatic hydrocarbons (PAHs) inducibility suggested that CYP1A1 was genetically regulated. Cytochrome CYP1A1 is a substrate-inducible microsomal enzyme that oxygenates PAHs generated from the combustion of fossil fuels, and aromatic amines, which are present in cigarette smoke, to carcinogens. In addition to cigarette smoke, inducers of the CYP1A1 enzyme include charcoal-broiled meat (a source of polycyclic aromatic hydrocarbons), cruciferous vegetables (a source of various indols), and omeprazole, a proton-pump inhibitor used to suppress gastric acid secretion. Reactions preferentially catalyzed by CYP1A1 include the hydroxylation and epoxidation of benzo[a]pyrene.

Hildebrand *et al*. (Hil85) assigned the *CYP1A1* gene to chromosome 15. Jaiswal and Nebert (Jai86) indicated that this locus is in the 15q22-qter segment. Several polymorphisms in the *CYP1A1* gene have been described so far but they are relatively rare in Caucasians and their functional significance remains unclear. The first polymorphism discovered involves the transition of thymidine to cytosine at position 3801 of genomic DNA, conferring a restriction endonuclease site for cleavage by *Msp*I (Spu87). This substitution occurs in the 3' noncoding region, downstream from exon 7 of the *CYP1A1* structural gene. A second polymorphism, an A>G substitution at position 2455 in exon 7 of the *CYP1A1* gene (Hay91a), results in an Ile to Val substitution at residue 462 near the heme binding region of the CYP1A1 protein. Another *Msp*I polymorphism (m3), which has been described by Crofts *et al.* (Cro93) at nt 3205, was shown to be African black-specific. Mutation m4 (2453C>A), causing threonine to asparagine substitution, was described by Cascorbi *et al*. (Cas96c). The *CYP1A1* promoter region contains at least three polymorphic sites (-4335G>A (Gai03)), -3229G>A, -3219C>T (Sma00)). These polymorphisms are located in the area of the gene which is well conserved between human and mouse (Jai85a).

Mutations of *CYP1A1* gene are presented in Figure 2.

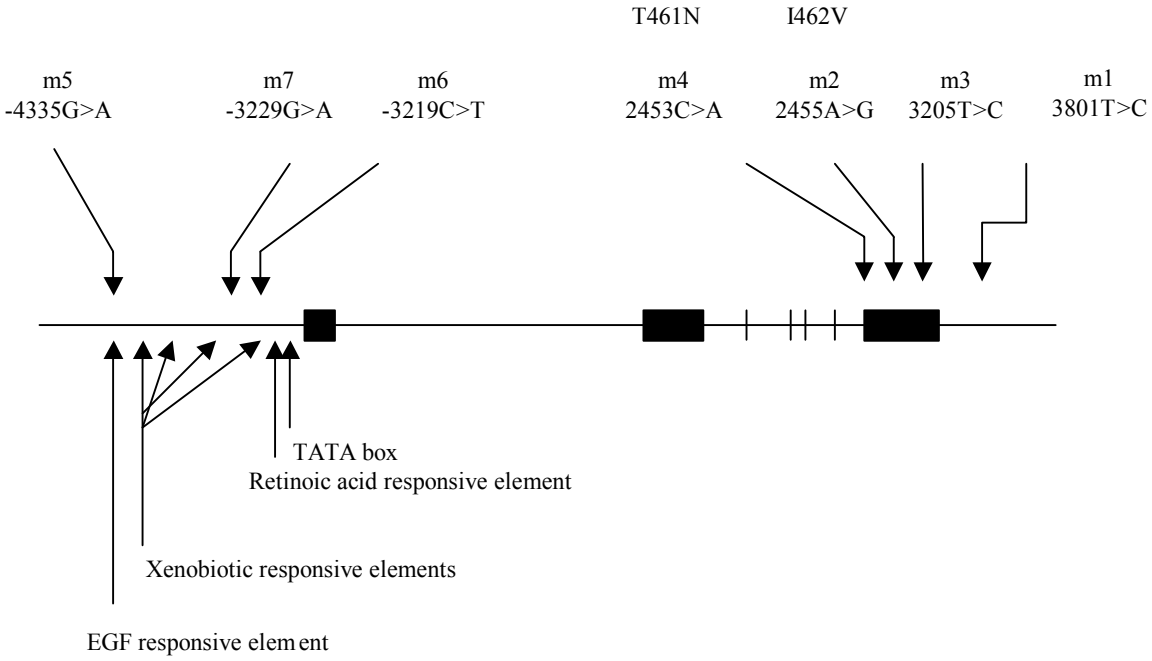


Figure 2: Polymorphisms in the human cytochrome P450 1A1. TATA box - the basal promoter contains a sequence of 7 bases (TATAAAA). EGF - epidermal growth factor.

Table 3: Alleles of *CYP1A1.*

m1 3801T>C	m2 2455A>G	m3 3205T>C	m4 2453C>A	m5 -4335G>A	m6 -3219C>T	m7 -3229G>A	Allele	References
T	A	T	C	G	C	G	**1A* (wt)	Jai85a, Jai85b, Kaw86
T	A	T	C	G	**T**	G	**1B*	Sma00
T	A	T	C	G	C	**A**	**1C*	Sma00
T	A	T	C	**A**	C	G	**1D*	Gai03
T	A	T	C	**A**	C	**A**	**1E*	Gai03
C	A	T	C	G	C	G	**2A*	Spu87
C	**G**	T	C	G	C	G	**2B*	Hay91b
T	**G**	T	C	G	C	G	**2C*	Hay91b, Zha96, Pers97
T	A	**C**	C	G	C	G	**3*	Cro93
T	A	T	**A**	G	C	G	**4*	Cas96c

In this study all known point mutations of the *CYP1A1* gene were investigated and defined to alleles.

1.1.3 Cytochrome P450 2D6 (CYP2D6)

It was shown in 1977 by Mahgoub *et al*. (Mah77) that a volunteer's hypotensive response to debrisoquine, a sympatholytic antihypertensive drug, was markedly increased because of impaired metabolism. In 1975, Eichelbaum (Eic75) observed increased side effects which were associated with decreased oxidative metabolism of sparteine, an alkaloid drug with antiarrhythmic actions. Family studies revealed that these two oxidative metabolic reactions are under monogenic control and that poor metabolizers are homozygous for a recessive allele.
Cytochrome P450 2D6 has been reported to catalyze the metabolism of about 25% of the clinically used drugs (Ben95), the most important of which are presented in Table 4.

Table 4: Substrates of CYP2D6 (according to Bertz & Granneman (Ber97); Tucker (Tuc94); Brockmöller (Bro00); Caccia (Cac98)).

Group of drugs	
Antiarrhythmic drugs	Ajmalin, flecainide, mexiletine, propafenone, sparteine
Tricyclic antidepressants	Amitriptyline, clomipramine, nortriptyline, fluoxetine, desipramine, fluvoxamine
Antihypertensives	Debrisoquine, indoramine
Beta-blockers	Metoprolol, propranolol, timolol
Neuroleptics	Haloperidol, remoxipride, risperidone
Opioide	Codeine, dextromethorphan, galanthamine, tramadol
Others	Methylendioxymetamphetamine ("ecstasy")

The *CYP2D* wild-type locus in humans is comprised of the three highly homologous genes, *CYP2D8P*, *CYP2D7P*, and *CYP2D6*, which are located in this orientation (5' to 3') on a contiguous region of about 45 kb on chromosome 22q13.1 (Gon88, Kim89). *CYP2D* genes consist of nine exons and eight introns (Figure 3). *CYP2D8P* and *CYP2D8P* were found to be pseudogenes.

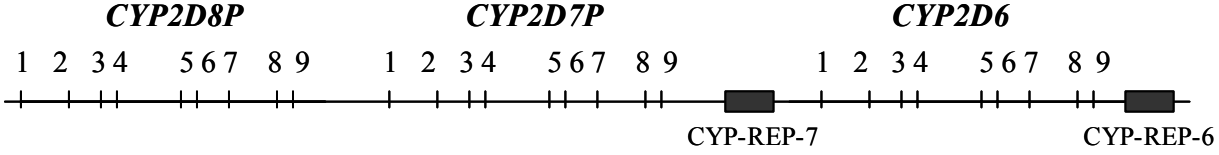


Figure. 3: Localization of *CYP2D6* gene and of pseudogenes *CYP2D7P* and *CYP2D8P* in the *CYP2D*-geneclaster in chromosome 22. Repetitive elements are shown as grey boxes.

Seventeen different alleles of *CYP2D6*, associated with deficient, reduced, or increased activity, are known. They are termed according to the unified nomenclature developed by Daly *et al*. (Daly96a). Most alleles consist of point mutations, but a deletion of the entire gene (allele **5*) and gene conversions, resulting in the «hybrid» alleles **13* and **16*, also exist (Pan95, Daly96b). There are gene duplications of the**2* allele (**2x2*; Joh93), as well as of the **1* and **4* alleles (Dah95, Lov96) and higher amplifications of the *CYP2D6* genes (Joh93, Akl96). The most frequent inactivating mutation in Caucasians is the splice-site mutation 1934G>A defining the *CYP2D6*4* allele (former type B allele), which results in a loss of enzyme activity. The alleles **2*, **9*, **10* and some others show slightly or moderately reduced activity in comparison with the wild-type allele **1*. The poor metabolizer status is genetically based on the presence of a combination of any of a number of defective alleles.

1.2 Phase II enzyme reaction

Phase II enzymes are sometimes involved in metabolic activation, but usually conjugate various phase I products and other reactive intermediates, completing the detoxification cycle. With the exception of methylation and acetylation, phase II biotransformation reactions result in a large increase in the hydrophilicity of the xenobiotic, so they greatly promote the excretion of exogenous substances. Most phase II biotransforming enzymes are located in the cytosol; notable exceptions are the UDP-glucuronosyltransferases, which are microsomal enzymes. Phase II reactions generally proceed much faster than phase I reactions, such as those catalyzed by cytochrome P450. Therefore, the rate of elimination of xenobiotics, the excretion of which depends on the biotransformation by cytochrome P450 followed by phase II conjugation, is generally determined by the first reaction. Many phase II drug metabolizing enzymes have been shown to be polymorphically expressed, such as the *N*-acetyltransferases 1 and 2 (NAT1 and NAT2), the glutathione *S*-transferases M and T (GSTM and GSTT), and the thiopurine *S*-methyltransferase (TPMT).

1.2.1 Arylamine *N*-acetyltransferase 2

N-acetylation is the major route of biotransformation for xenobiotics containing an aromatic amine (R-NH_2) or a hydrazine group (R-NH-NH_2), which are converted to aromatic amides (R-NH-$COCH_3$) and hydrazides (R-NH-NH-$COCH_3$), respectively (Eva92). Like methylation, *N*-acetylation masks the amine with a nonionizable group, so that many *N*-acetylated metabolites are less water soluble than the parent compounds. Nevertheless, *N*-acetylation of certain

xenobiotics such as isoniazid, facilitates their urinary excretion. In contrast to other xenobiotic-biotransforming enzymes, the number of *N*-acetyltransferases is limited (Vat95).

There exist two cytosolic *N*-acetyltransferases, namely NAT1 and NAT2. NAT1 is expressed in most tissues of the body, whereas NAT2 appears to be expressed only in the liver and the gut. NAT1 and NAT2 have different but overlapping substrate specificities, although no substrate is exclusively *N*-acetylated by one enzyme or the other. Substrates preferentially *N*-acetylated by NAT1 include *para*-aminosalicylic acid, *para*-aminobenzoic acid, sulfamethoxazole, and sulfanilamide, while substrates preferentially *N*-acetylated by NAT2 include isoniazid, hydralazine, procainamide, dapsone, aminoglutethimide, and sulfamethazine. Some xenobiotics, such as the carcinogenic aromatic amine, 2-aminofluorene, are acetylated equally well by NAT1 and NAT2.

The interindividual variation in NAT2 function is associated with the classical isoniazid acetylation polymorphism, which was discovered about fifty years ago by Bönicke and Reif (Bön53). As shown by the study of Evans *et al*. (Eva60), the «slow inactivator» person is homozygous for a slow inactivator allele, whereas the «rapid inactivator» is either homozygous or heterozygous for a rapid acetylator allele. Population studies show a distinct bimodal distribution of NAT2 phenotypes, as shown by Cascorbi *et al*. (Cas95), in 563 German subjects (Figure 4).

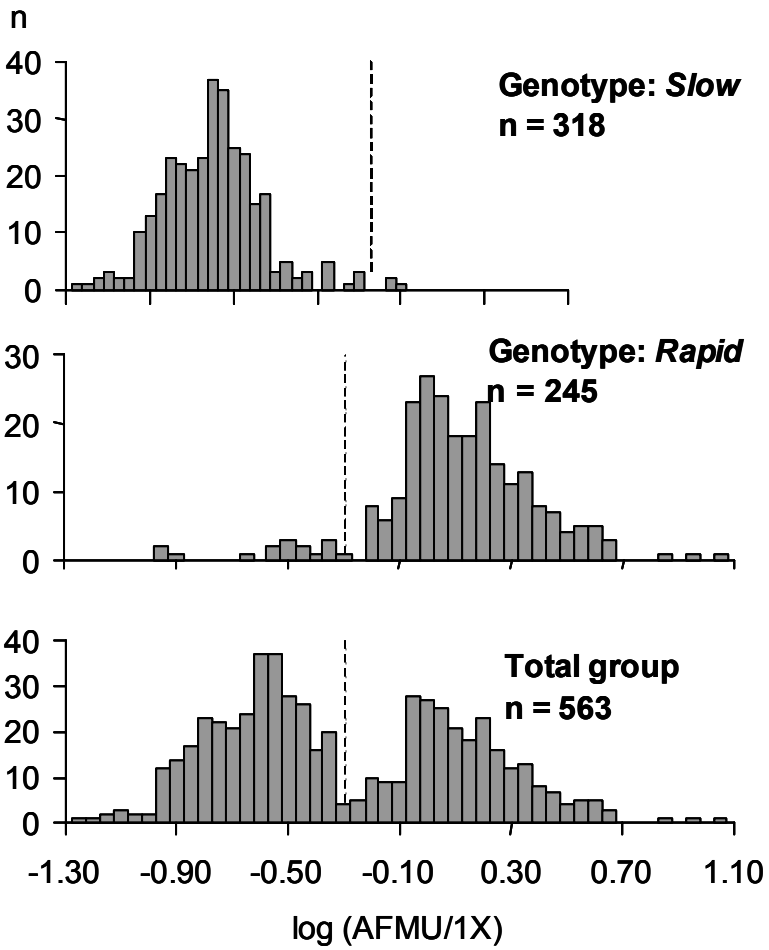


Figure 4: Histogram of NAT2 phenotypic activities as obtained by the caffeine test. Values represent the logarithmically transformed urinary metabolite ratio of 5-acetylamino-6-formylamino-3-methyluracil/ 1-methylxanthin (AFMU/1X) after the intake of caffeine (Cas95).

The association of the acetylation polymorphism with the risk of some kinds of malignant diseases (Eva92, Roo92) has received much attention. The slow acetylator phenotype was shown to be associated with a higher risk of bladder cancer (Car82, Ris95, Bro96), whereas the rapid acetylator phenotype is at a higher risk of developing colorectal cancer (Lan86, Pro95).
NAT1 and *NAT2* were mapped to chromosome 8p21.3-23.1 by Hickman *et al.* (Hic94). Each of the two genes *NAT1* and *NAT2* has a single, intronless protein-coding exon with an open reading frame of 870 bp. The nucleotide homology between *NAT1* and *NAT2* is 87% in the coding region. The pseudogene *NATP* has a high sequence homology to *NAT1* and *NAT2* but contains multiple frameshifts and stop codons and does not encode a protein (Blu90). Recent studies suggest that the NAT1 function may be genetically variable in human populations (Cri94,

Vat93). The *NAT2* gene was first described by Blum *et al.* (Blu90). Since then eight different point mutations have been characterized (Vat91, LinH94, Bel93, Lef99). Among the eight point mutations in the coding region are five which cause amino acid changes: 191G>A (Arg to Glu), 341T>C (Ile to Thr), 590G>A (Arg to Gln), 803A>G (Lys to Arg), 857G>A (Gly to Glu), and three silent ones: 111T>C, 282C>T and 481C>T (Figure 5).

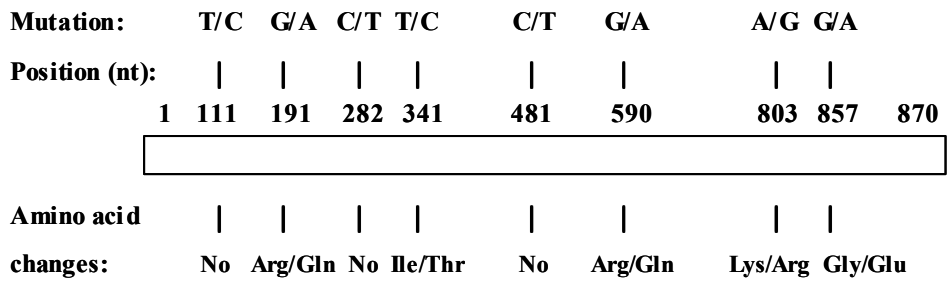


Figure 5: Polymorphism in the human *NAT2* gene.

The point mutations of *NAT2* form different alleles, which are presented in Table 5. Three nomenclatures of *NAT2* alleles have been used so far. The M1 allele has mutations at positions 341 and 481, M2 at positions 282 and 590, and M3 at position 857. According to the nomenclature of Vatsis *et al*. (Vat95), M1 corresponds to *NAT2*5A*, M2 to *NAT2*6A*, and M3 to *NAT2*7A*. Initially, only one mutation was reported at position 857 for *NAT2*7A*. Vatsis *et al*. (Vat95) described an allele, containing mutations at positions 341, 481 and 803 (*NAT2*5B*). Hickman *et al*. (Hic92) observed an allele with mutations at positions 341 and 803 (*NAT2*5C*). Moreover, Bell *et al*. (Bel93) described a mutation at position 191 that occurs almost exclusively in Blacks. The novel *NAT2*6D* allele was described by Leff *et al*. (Lef99).

Table 5: Alleles of *NAT2*.

NAT2-allele	Nucleotide positions of point mutations							
	111	191	282	341	481	590	803	857
*NAT2*4*	T	G	C	T	C	G	A	G
*NAT2*5A*	T	G	C	**C**	**T**	G	A	G
*NAT2*5B*	T	G	C	**C**	**T**	G	**G**	G
*NAT2*5C*	T	G	C	**C**	C	G	**G**	G
*NAT2*6A*	T	G	**T**	T	C	**A**	A	G

*NAT2*6D*	**C**	G	**T**	T	C	**A**	A	G
*NAT2*7B*	T	G	**T**	T	C	G	A	**A**
*NAT2*12A*	T	G	C	T	C	G	**G**	G
*NAT2*14A*	T	**A**	C	T	C	G	A	G
*NAT2*14B*	T	**A**	**T**	T	C	G	A	G

The wild-type *NAT2*4* (Blu90) allele and the rare *NAT2*12A* (Cas96a) allele are associated with the rapid acetylator phenotype. Eight alleles of the *NAT2* gene are coding for the slow phenotype. The In-vivo acetylation capacity is significantly higher in homozygous wild-type subjects than in heterozygous genotypes.

1.3 The purpose of the work

1. The purpose of this work was the investigation of the frequency of functionally important mutations, alleles and genotypes of the polymorphic cytochromes P450 *1A1* and *2D6* and arylamine *N*-acetyltransferase 2 in a group of 325 healthy Russian volunteers and the comparison of our data with corresponding frequencies of other ethnic groups.
2. In addition, a real time PCR method, which combines a microvolume fluorimeter with a rapid temperature cycler, was optimized for the detection of the *NAT2* polymorphism. This method is easy, reliable and fast, and allows to genotype hundreds of samples in one day.
3. The results of the study should provide the basis of a pharmacogenetic databank for the Russian population, which is the biggest Slavic group.

2 Materials and methods

2.1 Patients

We have investigated 325 (174 males, 151 females) unrelated individuals from the European part of Russia (Voronezh and region). The mean age (±SD) was 40.9±16.4 years (range 14-77). Except for 76 healthy volunteers, subjects were outpatients with non-malignant diseases recruited from the departments of otorhinolaryngology (158), surgery (47), neurology (41) and traumatology (3) (Table 6) at the Central Railway Clinic in Voronezh. All subjects were informed about the contents and aims of the study and gave their written consent. When the participants were under 18 years old, the written consent was given by their parents. Information about present or former smoking habits was collected from all the participants. The study was approved by the Central Moscow Ethics Committee.

Table 6: Characteristics of study participants.

Participants	n	Age range (years)	Mean age (years)	Median age (years)	Percentiles	
					25	75
Total	325	14-77	41.6 ± 16.4	41.0	25.0	54.0
Males	174	15-73	38.7 ± 16.2	38.0	23.0	51.3
Females	151	14-77	43.3 ± 16.2	45.0	29.0	57.0
Healthy volunteers	76	18-61	27.4 ± 10.9	23.0	21.0	28.3
Dept. of otorhinolaryngology	158	14-74	43.0 ± 15.8	43.0	31.0	54.8
Dept. of surgery	47	16-77	50.2 ± 15.2	52.0	37.0	62.0
Dept. of neurology	41	21-67	47.6 ± 12.9	49.0	42.0	57.0
Dept. of traumatology	3	44-72	55.0 ± 14.9	49.0	44.0	72.0

2.2 Materials

2.2.1 Chemicals

The following chemicals and solutions were used for DNA extraction and molecular genetic investigations.

Table 7: Chemicals used for investigation.

Chemicals	Manufacturer
DNA polymerase *AmpliTaq*™,	Perkin Elmer
10x PCR buffer, 25 mM $MgCl_2$	
DNTPs	Boehringer Mannheim
Restriction endonucleases	New England Biolabs
*Kpn*I, *Bam*HI, *Taq*I, *Msp*I, *Fok*I, *Dde*I,	
*Bst*NI, *Ban*II, *Hph*I,	
*Bsr*DI, *Bsa*I, *Hha*I, *Bsl*I, *Nco*I	
DNA oligonucleotides (PCR primers)	TIB-Molbiol
Hybridization probes	TIB-Molbiol
100 bp and 1 kb DNA marker	MBI Fermentas
marker V and marker VI	Boehringer Mannheim
Proteinase K	Boehringer Mannheim
Agarose ultrapure, agarose NuSieve	Gibco BRL, Biozym
Expand™ Long PCR System	Boehringer Mannheim

Table 8: Solutions used for DNA extraction and gel preparation.

Solution	Contents
Lysis buffer	NH_4Cl (155 mM), $KHCO_3$ (10 mM), EDTA (0.1 mM); pH 8.0
TEN-buffer	Tris/HCl (20 mM), EDTA (2mM), NaCl (30 mM); pH 7.5
20% SDS	2 mg sodium dodecylsulfate (Merck) in 100 ml H_2O
Phenol solution	Phenol/chloroform/water (Perkin Elmer)
Chloroform solution	Chloroform, isoamylic alcohol (49:1)
Sodium acetate	40.8 g CH_3COONa (3 M) fill up ad 100 ml H_2O; pH 5.5
TE buffer	Tris-HCl (10 mM), EDTA (1mM) fill up ad 100 ml H_2O; pH 8.0
TBE buffer	Tris (90 mmol/l), boric acid (90 mmol/l), EDTA (2.5 mmol/l); pH 8.0-8.3

2.2.2 Equipment

The experimental part of the work was done in the Institute of Clinical Pharmacology, Charité, Berlin. The equipment used is listed in Table 9.

Table 9: Instruments used for experiment.

Instruments	Manufacturer
Thermocyclers: Gene Amp PCR System 9600 and 9700	Perkin Elmer/Applied Biosystems
LightCycler™	Roche Diagnostics Inc.
Video detection system Eagle Eye	Stratagene
Incubation ovens	Biometra
Centrifuges	Eppendorf, Beckman, Sigma
Several semi-automatic shakers	Hoefer, Heidolf, Janke & Kunkel
Electrophoresis chambers and Electrophoresis power supplies	Pharmacia, Biorad, Hoefer

2.3 Methods

2.3.1 DNA extraction

DNA was extracted manually by the phenol-chloroform method, according to Sambrook *et al*. (Sam89). Ten ml of blood were taken from each patient and mixed with a solution of ethylene diamine tetraacetic acid (1.6 mg EDTA/ml of blood). Then 40 ml of 1x lysis buffer were added and the sample was incubated in ice for 30 min. Samples were centrifuged at 2000 rpm at 4°C for 30 min. The supernatant liquid was removed, the cell pellet was dissolved in 1.5 ml 1x TEN buffer and stored at -20°C. For the genotyping procedure, the samples were transported in dry ice to Berlin. After thawing the cell suspension was mixed with 100 µl 20% SDS solution and 100 µl proteinase K solution and, being shaken at 40 1/min, incubated at 37°C overnight. Next day 1.5 ml of phenol were added to the proteinase-digested cell suspension which was then shaken for 3 hours in an overhead-shaker at 30 1/min. Then tubes were centrifuged at 3000 rpm for 5 min to separate phases. The upper, DNA containing phase, was transferred into a 12-ml reaction tube. In the next extraction step lipids were removed from the DNA solution by adding 1.5 ml chloroform solution and shaking in an overhead shaker (30 1/min) for 1 hour. Phases were

separated by centrifugation at 3000 rpm for 5 min. The DNA containing upper phase was carefully transferred into fresh 12-ml tubes. DNA was precipitated by adding 100 µl of 3 M sodium acetate (pH 5.5) and 6 ml of 96% ethanol, tubes were mixed well and centrifuged (10 min 3000 rpm). All supernatant liquid was removed. Three ml of 70% ethanol were added to the DNA, mixed well and centrifuged (10 min 3000 rpm). The supernatant liquid was removed and DNA was dried by placing the tubes overhead on filter paper for 10 min. Dried DNA was dissolved in 600 µl TE buffer and incubated at 55°C overnight, simultaneously slightly agitated at 40 1/min. On the next day the DNA solution was transferred into labelled 1.5-ml sterile Eppendorf reaction tubes and samples were stored at 4°C. DNA concentrations were measured with a spectrophotometer (Eppendorf, Germany) and, when necessary, DNA was diluted to the concentration of 30 ng/µl.

2.3.2 Genotyping methods

2.3.2.1 Polymerase chain reaction/ restriction fragment length polymorphism (PCR-RFLP)

The investigation of genes encoding drug metabolizing enzymes was performed using polymerase chain reaction (PCR). The main principle of PCR is the exponential amplification of the part of DNA which contains the gene of interest. The amplification product can be analyzed for genomic alterations (mutations, deletions, translocations). For successful PCR, about 50-60 ng DNA, DNA-polymerase, deoxyribonucleotide triphosphates (dATP, dCTC, dGTP and dTTP) and two specific primers should be incubated under specific conditions. The PCR procedure includes three consecutive phases. During the first phase the DNA is denatured by heating to 95°C. In the next phase (annealing, 55-65°C), primers hybridize to the complementary parts of single-strand DNA-matrix. During the third part of PCR (extension, 72°C), the formation of the second strand is completed. In the following cycles, the newly formed molecules of DNA are used as matrix. The number of cycles in one PCR is 25-35, these are necessary to obtain enough genetic material for further analysis. Five µl of PCR-product are analyzed in a 1% agarose gel. Positive samples can be used for further investigations. The PCR-product is digested using restriction endonucleases, which results in fragments the lengths of which depend on the genotype. Heterozygous individuals have fragments typical for both wild-type and mutant alleles. The digest is mixed with 10 µl of blue buffer (Life Technologies) and fragments are separated in the 2-4% gel. 4.5µl of DNA-marker are used as a size standard of fragments. Results are documented using the video detection system Eagle Eye.

2.3.2.2 LightCycler assay

The hybridization probe format is used for DNA detection and quantification and provides maximum specificity for product identification. In addition to the reaction components used for conventional PCR, two specially designed, sequence-specific oligonucleotides labelled with two different fluorescent dyes are utilised for this detection method. The detection is based on the generation of a fluorescent signal by fluorescence resonance energy transfer (FRET), which is released when two probes bind to the target sequence.

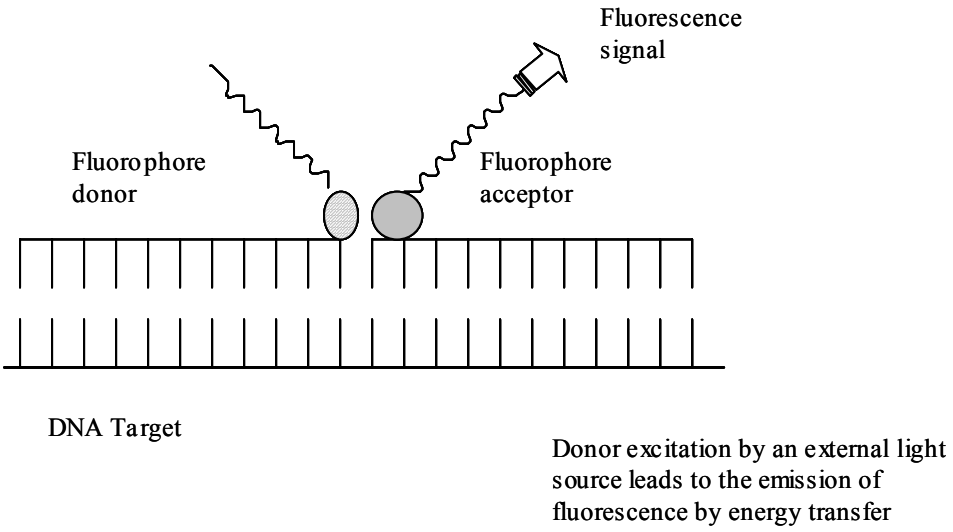


Figure 6: PCR monitoring with hybridization probes.

The LightCycler method allows highly specific detection of the amplification product as described below. Figure 6 shows the three essential components for using fluorescence-labelled oligonucleotides as hybridization probes: two differently labelled oligonucleotides and the amplification product. Oligo 1 carries a fluorescein label at its 3' end whereas oligo 2 carries different label (LC Red 640 or LC RED 705) at its 5' end. The sequences of these two oligonucleotides are selected in such a way that they hybridize to the amplified DNA fragment in a head to tail arrangement. When the oligonucleotides hybridize in this orientation, the two fluorescence dyes are positioned in close proximity to each other. The first dye (fluorescein) is excited by the LightCycler's LED (Light Emitting Diode) filtered light source, and emits green fluorescent light at a slightly longer wavelength. When the two dyes are in close proximity, the emitted energy excites the LC Red 640 attached to the second hybridization probe that subsequently emits red fluorescent light at an even longer wavelength. This energy transfer,

referred to as FRET, is highly dependent on the spacing between the two dye molecules. Only if the distance between the two molecules does not exceed 5 nucleotides is the energy transferred very efficiently. When the appropriate detection channel is chosen, the intensity of the light emitted by the LightCycler – Red 640 is filtered and measured by the LightCycler's optics. The increasing amount of measured fluorescence is proportional to the increasing amount of DNA generated during the ongoing PCR process. Since LC Red 640 only emits a signal when both oligonucleotides are hybridized, the measurement of fluorescence is performed after the annealing step.

2.3.2.3 Genotyping of *CYP1A1* mutations

Seven polymorphic sites of the polymorphic *CYP1A1* were analyzed.

PCR No. 1 Amplification of a 899-bp fragment (detection of mutations m1 and m3).

An 899-bp fragment, containing polymorphic sites m1 and m3, was amplified with the forward primer M3F and the reverse primer P80 (Table 10). PCR reactions included 2.5 µl PCR-buffer, 2.5 µl 2 mmol/l dNTPs, 0.5 µl of each of the primers, 0.5 U *Taq*-DNA-polymerase, 2.4 µl 25 mmol/l $MgCl_2$ and 50 ng of genomic DNA in a total volume of 25 µl. Amplification was performed with an initial denaturation at 94°C for 2 min, 35 cycles with at 94°C for 30 s, at 63°C for 30 s, at 72°C for 1 min, and a final elongation at 72°C for 7 min.

The PCR-product, containing an 899-bp fragment, was digested with restriction enzyme *Msp*I at 37°C overnight.

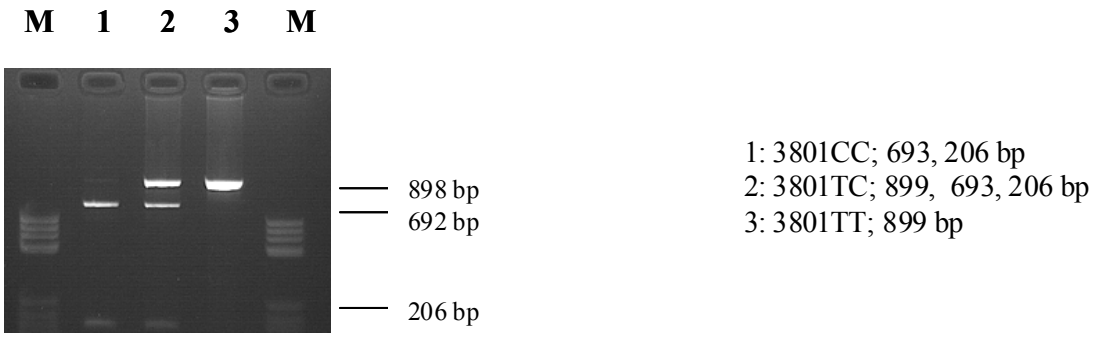


Abb. 7: *Msp*I digest of an 899-bp fragment containing the positions of the m1/m3 mutations.

Fragments were colored with 1mg/l Ethidium Bromide.

PCR No. 2. Amplification of a 204-bp fragment (detection of mutations m2 and m4).

For the detection of the m2 and m4 mutations, a 204-bp fragment was amplified with the primers M2F and M2R. A 50-µl PCR mix contained 5 µl PCR- buffer, 5 µl 2 mmol/l dNTPs, 4.8 µl 25 mmol/l $MgCl_2$, 1 µl of each of the primers, 1 U *Taq*-polymerase, 35.4 µl H_2O and 50 ng of genomic DNA. Thermocycling conditions were the same as for the PCR No.1. *Bsr*DI and *Bsa*I were used as restriction enzymes for m2-digestion and for m4-digestion, respectively. The restriction-fragment lengths are given in Table 11. The digestion products were analyzed on a 3% agarose gel, together with a 100-bp DNA weight marker.

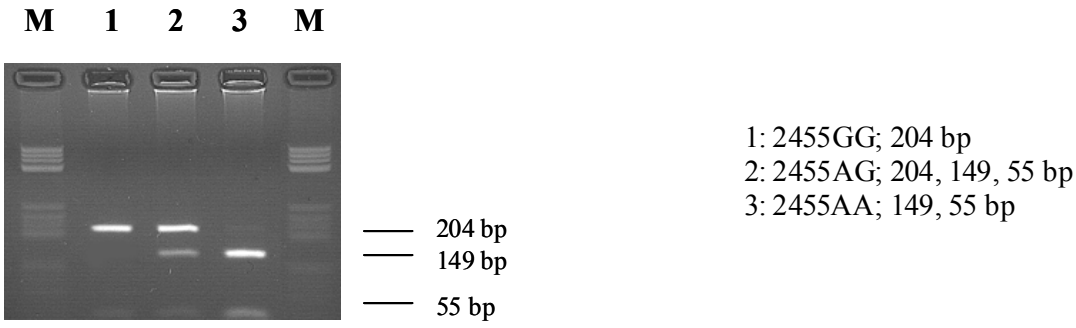


Figure 8: *Bsr*DI digest of a 204-bp fragment containing the positions of the m2 mutation.

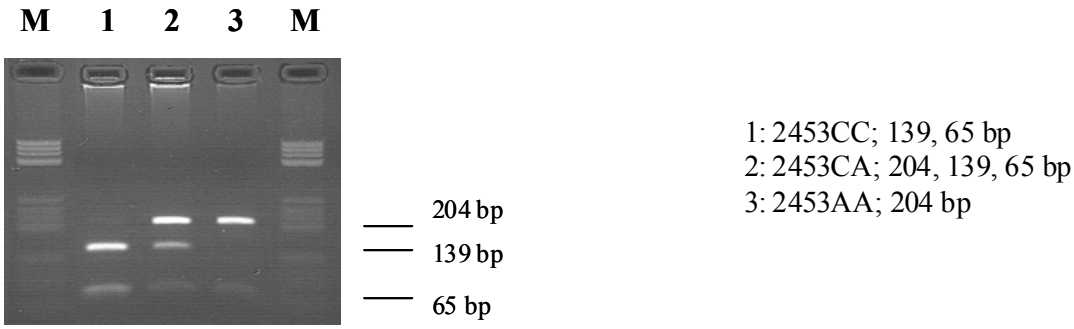


Figure 9: *Bsa*I digest of a 204-bp fragment containing the positions of the m4 mutation.

PCR No. 3 Amplification of a 157 bp-fragment (detection of the new polymorphism m5).

PCR with the primers Hha-F and Hha-R resulted in the amplification of a 157-bp fragment. The mastermix contained 2.5 µl PCR-buffer, 2.5 µl 2 mmol/l dNTPs, 1 µl of each of the primers, 0.5 U *Taq*-polymerase, 2 µl 25 mmol/l $MgCl_2$, 15.9 µl H_2O, and 50 ng of genomic DNA. PCR-conditions were as follows: an initial denaturation at 94°C for 2 min, 35 cycles at 94°C for 20 s,

at 55°C for 10 s, at 72°C for 40 s and at 72°C for 7 min. The PCR-product was digested with *Hha*I at 37°C for 2 hours.

PCR No.4 Amplification of a 284-bp fragment (detection of the mutations m6 and m7).
For the detection of possible m6 and m7 mutations, a PCR-amplified 284-bp fragment was used. A 50-µl PCR mix contained 5 µl PCR-buffer, 5 µl 2 mmol/l dNTPs, 1 µl of each of the primers 1A1-M5F and 1A1-M5R, 1 U *Taq*-polymerase, 4 µl 25 mmol/l $MgCl_2$, 33.8 µl H_2O and 50 ng of genomic DNA. The amplification was performed with an initial denaturation at 94°C for 2 min, 35 cycles at 95°C for 30 s, at 60°C for 1 min, at 72°C for 30 s, and a final elongation at 72°C for 7 min. The PCR-product, containing a 284-bp fragment, was subject to digestion with enzymes *Bsl*I and *Nco*I for the detection of m6 and m7 mutations.

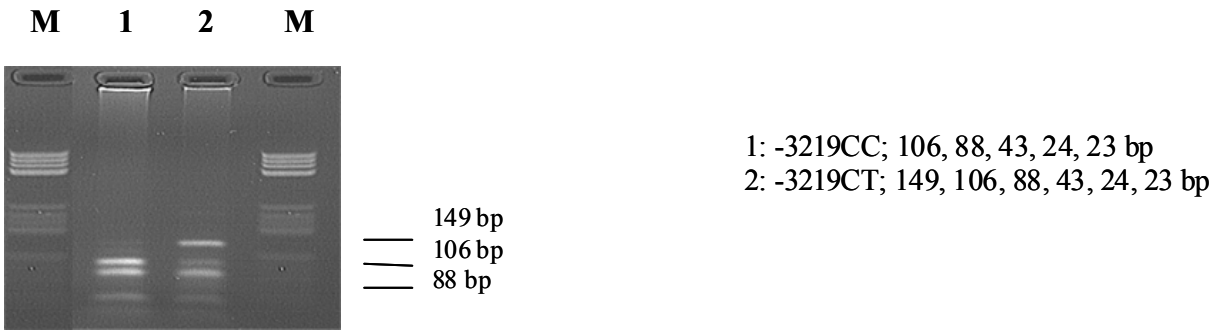


Abb. 10: *Bsl*I digest of a 284-bp fragment containing the positions of the m6 mutation.

Table 10: Primers for *CYP1A1* genotyping.

Primer	Fragment length (bp)	Sequence
M3F	899	5'-GGCTGAGCAATCTGACCCTA
P80	-	5'-TAGGAGTCTTGTCTCATGCCT
M2F	204	5'-CTGTCTCCCTCTGGTTACAGGAAGC
M2R	-	5'-TCCCACCCGTTGCAGCAGGATAGCC
Hha-F	157	5'-TGGGGCATATTACTTGTCTCCTT
Hha-R	-	5'-CGGCCTCGTGCATTGCAGAAATA
1A1-M5F	284	5'-GAACCTCAGCTAGTCGCCC
1A1-M5R	-	5'-AGAGAGGGTACGGGAAGCTC

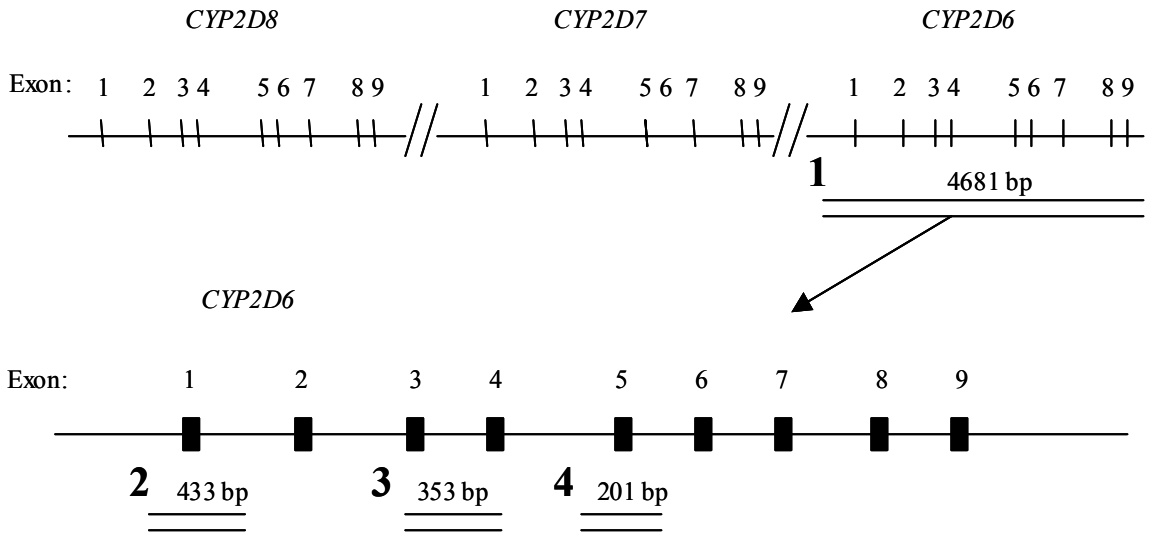
Table 11: Restriction endonucleases for recognition of *CYP1A1* mutations.

Mutation	Position	Transition	Restriction endonuclease	Recognition sequence	Preamplification with primers	Fragment-length (bp) Wild type* Mutation
m1	3801	T>C	*Msp*I	C^CGG	M3F/P80	899 693 206
m2	2455	A>G	*Bsr*DI	GCAATGnn^	M2F/M2R	149 55 204
m3	3205	T>C	*Msp*I	C^CGG	M3F/P80	899 802 97
m4	2453	C>A	*Bsa*I	GGTCTCn^nnnn	M2F/M2R	139 65 204
m5	-4335	G>A	*Hha*I	GCG^C	HhaI-F/ HhaI-R	94 63 157
m6	-3219	C>T	*Bsl*I	CCnnnnn^nnGG	1A1-5F/ 1A1-5R	106 88 43 24 23 149 88 24 23
m7	-3229	G>A	*Nco*I	C^CATGG	1A1-5F/ 1A1-5R	284 141 143

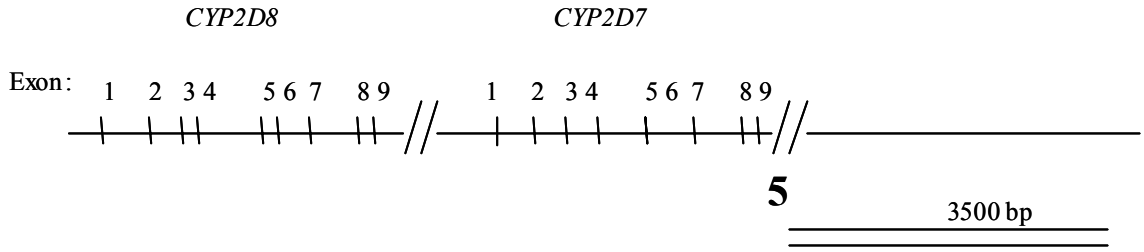
*First line, wild-type fragment; second line, mutant fragment

2.3.2.4 Genotyping of *CYP2D6* mutations

For the detection of the most functionally important alleles **1,*3,*4,*5,*6,*10* and the gene duplication of *CYP2D6*, combined PCR-RFLP tests were used, as described by Sachse *et al*. (Sac97). The first step was the amplification of a 4681-bp genomic DNA fragment, which contained all nine *CYP2D6* exons, using the Expand Long Template PCR System™. Figure 11 shows consecutive steps of *CYP2D6* genotyping.



Detection of *CYP2D6*5* deletion



Detection of *CYP2D6*MxN* duplication

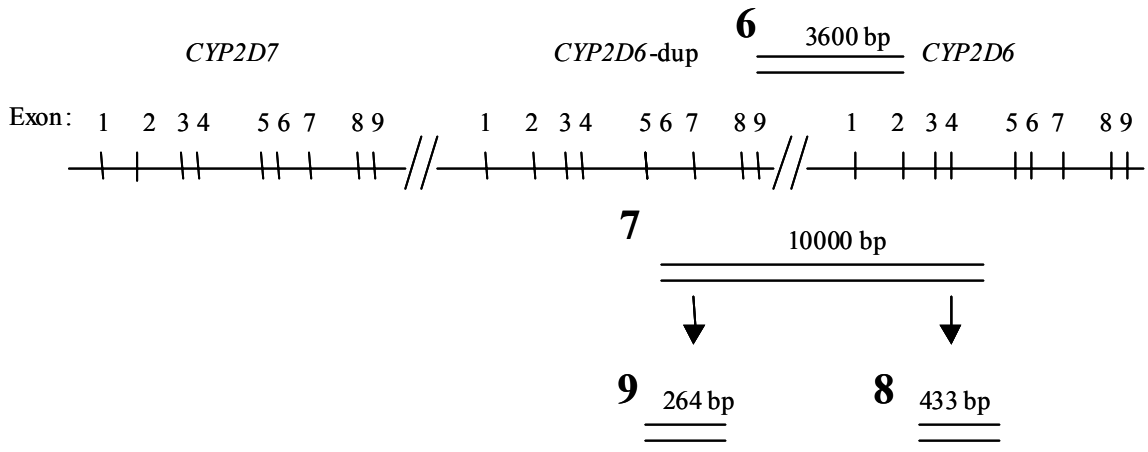


Figure 11: Design of the PCR tests for *CYP2D6* mutations. The bold numbers indicate the RCP reactions which are described in the text.

Table 12: PCR-RFLP tests for *CYP2D6* genotyping.

PCR No.	Detected mutation	PCR-RFLP assay			RFLP-fragment patterns [bp]	
		PCR primers	Fragment length [bp]	Restriction enzyme	Wild-type allele	Mutant allele
1	entire *CYP2D6*	P100/P200	4681	-	4681	no fragment in cases of *5/*5
2	188C>T	P113/P121	433	*Hph*I	362/71	262/100/71
3	1795T>Del 1934G>A	P*3/P2	353	*Bst*NI *Bst*NI	190/163 190/163	190/139/123 353
4	2637A>Del	P51/D2	201	*Bsa*AI	201	180/20
5	**5*	P13/P24/P81	3500	-	4500	3500
6	*CYP2D6*MxN*	P17/P32	3600	-	5200	5200/3600
7	*CYP2D6*MxN*	P2x2f/P2x2r	10000	-	no fragment	10000
8	*CYP2D6*4xN*	P113/P121	433	*Hph*I	**1, *2*: 362/71	**4*: 262/100/71
9	*CYP2D6*1xN*	P2x2f/P92	264	*Ban*II	**1*: 231/33	**2, *4*: 264

PCR No.1 Amplification of the entire *CYP2D6* gene.

The amplification of the whole *CYP2D6* gene (4681-bp fragment) was performed according to Sachse *et al*. (Sac97). A 25-µl PCR mix contained 2.5 µl PCR-buffer, 4.5 µl 2 mmol/l dNTPs, 0.5 µl of each of the primers P100 and P200 (all primers are given in Table 13), 1.25 U *Taq*-polymerase, 16.75 µl of H_2O, and 80-100 ng of genomic DNA. Thermocycling conditions were as follows: initial denaturation at 94°C for 2 min, 35 cycles of denaturation at 96°C for 10 s, annealing at 57°C for 20 s, and extension at 68°C for 5 min. The terminal elongation was performed at 68°C for 7 min. If the PCR was successful (checked by 1% agarose gel electrophoresis), 15 µl of the PCR product were diluted with 5 volumes of distilled water and stored at 4°C.

PCR No. 2 Detection of the mutation 188C>T (alleles **4* and **10*).

Nested PCR was performed using the PCR product of reaction No. 1. A 433-bp fragment was amplified at 94°C for 2 min, 25 cycles at 95°C for 30 s, at 58°C for 10 s, at 72°C for 1 min, and terminal extension at 72°C for 7 min. A 25-µl PCR mix contained 2.5 µl PCR-buffer, 2.5 µl 2 mmol/l dNTPs, 1.25 µl 25 mmol/l $MgCl_2$, 0.5 µl of each of the primers P113 and P121, 1.25

U *Taq*-polymerase, 17.5 µl H_2O, and 1 µl of the diluted 4681 PCR-product. The PCR-product was digested with enzyme *Hph*I at 37°C overnight.

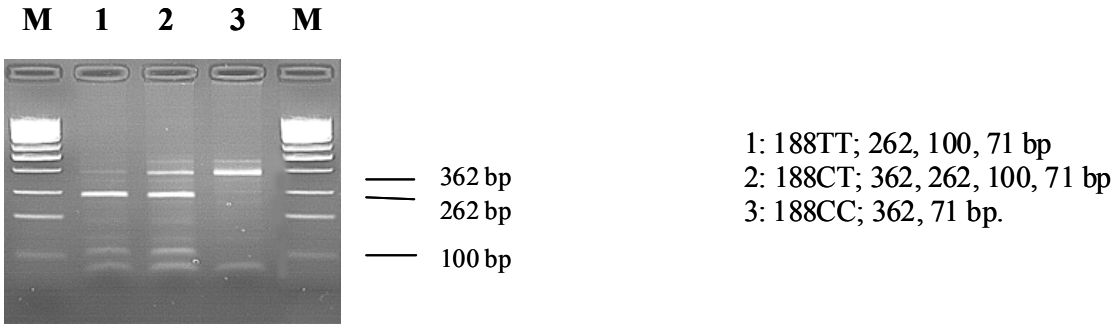


Figure 12: *Hph*I digest of a 433-bp fragment containing position 188 of the *CYP2D6* gene.

PCR No. 3 Detection of mutations 1795 T>Del and 1934G>A (alleles **4* and **6*).

PCR No. 4 Detection of mutations 2637A>Del (allele **3*).

In the subsequent nested PCRs, conditions were as follows: initial denaturation at 94°C for 2 min, 25 cycles at 95°C for 10 s, at 60°C for 10 s, at 72°C for 1 min, and terminal extension at 72°C for 7 min. A 25-µl PCR mix contained 2.5 µl PCR- buffer, 2.5 µl 2 mmol/l dNTPs, 1.25 µl 25 mmol/l $MgCl_2$, 0.5 µl of each of the primers, 1.25 U *Taq*-polymerase, 17.5 µl H_2O and 1 µl of the diluted 4681 PCR-product. After amplification, the products of nested PCRs were analyzed by an 1% agarose gel electrophoresis and digested with the respective restriction endonucleases. The enzymes and restriction-fragment lengths are given in Table 12. The digestion products were analyzed on a 3% agarose gel, together with a 100-bp DNA weight marker.

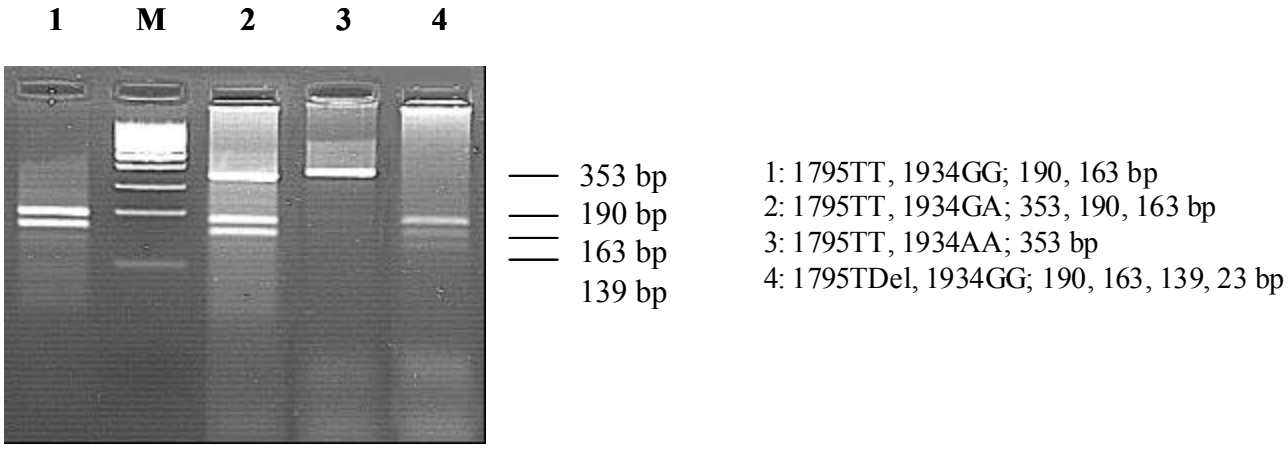


Figure 13: *Bst*NI digest of a 353-bp fragment containing the mutations 1795T>Del and 1934G>A of the *CYP2D6* gene.

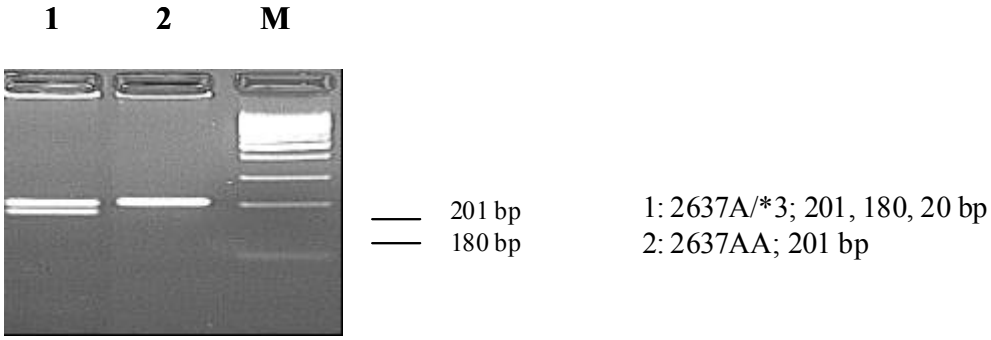


Figure 14: *Bsa*AI digests of a 201-bp fragment for the detection of the mutation 2637A>Del.

PCR No. 5 Detection of *CYP2D6* deletion (allele **5*).

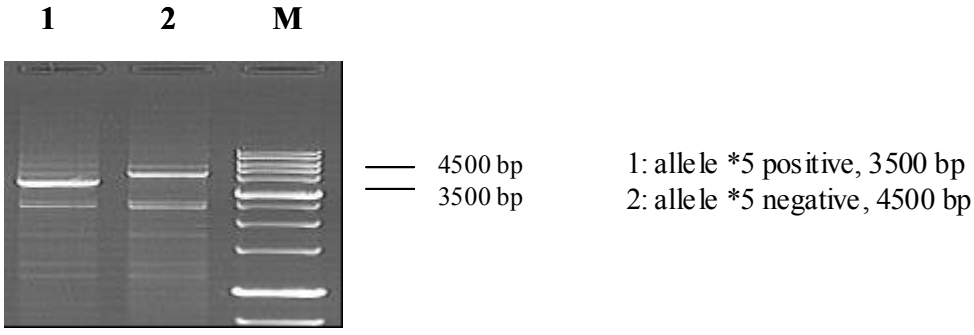


Figure 15: Electrophoresis of the PCR-product from reaction No. 5 for detection of the *CYP2D6* deletion.

PCR No. 6 Detection of *CYP2D6* duplication (allele **MxN*).

For the detection of the *CYP2D6* deletion (allele **5*) and duplication (**MxN*), PCR methods were established from literature. The *CYP2D6* deletion detecting assay (Ste95) was improved, according to Sachse *et al.* (Sac97), by adding an internal standard primer. This primer enables genotype independent amplification of an internal standard fragment, as in the *CYP2D6* duplication assay of Løvlie *et al*. (Lov96). These internal standard fragments ensure that false negative results are not possible in these two assays. In both PCRs, a standard fragment, and – if the deletion or duplication allele occurs – a second fragment are amplified from genomic DNA. After having transferred the product of the enzymatic digestion in 1% agarose gel, it was submitted to electrophoresis, and the results were documented by a digital video system. Long distance PCR technique was applied using the same concentrations of PCR compounds and PCR conditions as described above for the PCR reaction amplifying the entire *CYP2D6*.

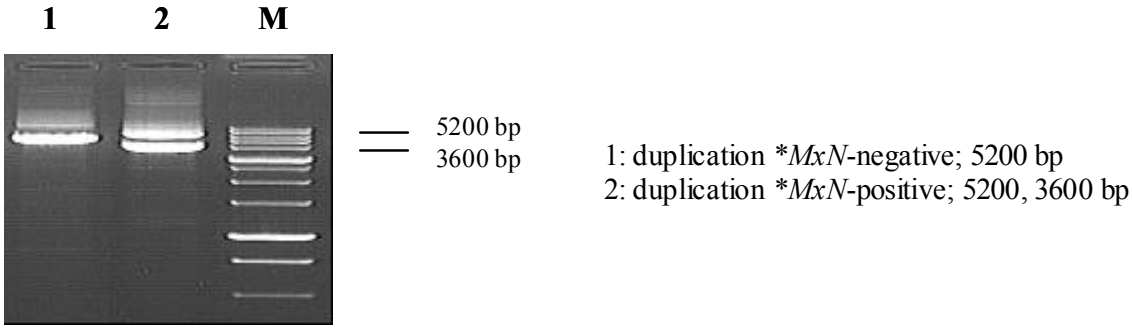


Figure 16: Electrophoresis of the PCR-product from reaction No. 6 for detection of the *CYP2D6* duplication.

PCR No. 7 Detection of *CYP2D6* duplication (allele **MxN*).

In 3 cases of allele duplication and genotype **1/*4*, PCR No. 7 was established according to Johansson *et al*., 1996 for the detection of duplicated allele. The PCR conditions were as follows: an initial denaturation at 94°C for 2 min, 10 cycles at 96°C for 10 s, at 57°C for 20 s, at 68°C for 12 min, 20 cycles at 96°C for 10 s, at 57°C for 20 s, at 68°C for 12 min + 15 s per cycle and terminal extension at 68°C for 7 min. A 25-µl PCR mix contained 2.5 µl PCR-buffer, 6.25 µl 2 mmol/l dNTPs, 0.75 µl of each of the primers P2x2f and P2xr, 1.85 µl of enzyme mix (*Taq* and *Pwo* polymerases), 11.5 µl H_2O and 80-100 ng of genomic DNA. The PCR resulted in a 10000-bp fragment, which is a fragment between exon 9 of allele 1 and intron 2 of allele 2.

PCR No. 8 Detection of *CYP2D6* duplication (allele **4xN*).

PCR No. 9 Detection of *CYP2D6* duplication (allele **1xN*).

To distinguish between different types of allele duplication, two additional PCR-RFLP tests were developed, both as nested PCR from the diluted product of reaction 7. A PCR-RFLP test detecting the **2*-related 4268G>C mutation (reaction 8) showed whether **1* or **2* was duplicated (in **1x2/*2* or **2x2/*1* constellation). The primers P2x2f and P92 were used to amplify a 264-bp product (PCR conditions as for reaction 3), which was digested using *Ban*II.

Performing reaction 9 (same conditions as in reactions 3 and 8) using the 10-kb gene duplication product as a target, the detection of the **4*-associated 188C>T mutation by *Hph*I digestion served as an indirect proof of a duplicated **4* allele in cases of a questionable **2x2/*4* or **4x2/*2* constellation.

Table 13: Sequences and locations of primers used in PCR reactions.

Primer	Sequence	Orientation and position*			Specificity
P100	5'-GGCCTACCCTGGGTAAGGGCCTGGAGCAGGA	f	-180	-150	whole *CYP2D6* gene
P200	5'-CTCAGCCTCAACGTACCCCTGTCTCAAATGCG	r	+92	+123	
P*3	5'-CCTGGGCAAGAAGTCGCTGGACCAG	f	1770	1794	alleles **4* and **6*
P2	5'-GAGACTCCTCGGTCTCTCG	r	2104	2122	
P51	5'-GCTGGGGCCTGAGACTT	f	2457	2473	allele **3*
D2	5'-GGCTGGGTCCCAGGTCATAC	r	2638	2657	
P13	5'-ACCGGGCACCTGTACTCCTCA	f (*2D7*)	+1619	+1639	
P24	5'-GCATGAGCTAAGGCACCCAGAC	r	+3444	+3465	allele **5*
P81	5'-CGTCTAGTGGGGAGACAAAC	f	3621	3640	
P17	5'-TCCCCCACTGACCCAACTCT	f (*2D7*)	+155	+174	*CYP2D6* duplication (allele **MxN*)
P32	5'-CACGTGCAGGGCACCTAGAT	r (*2D7*)	+5470	+5489	
P2x2f	5'-GCCACCATGGTGTCTTTGCTTTC	f	4238	4260	
P2x2r	5'-ACCGGATTCCAGCTGGGAAATG	r	1384	1405	
P113	5'-TCAACACAGCAGGTTCA	f	-82	-66	alleles **10*, **4xN*
P121	5'-CTGTGGTTTCACCCACC	r	335	351	
P92	5'-CTCAGCCTCAACGTACCCCT	r	+104	+123	allele **1x2*

Underlined nucleotides refer to mismatched bases

*Position according to Kimura *et al*. (Kim89)

2.3.2.5 Genotyping of *NAT2* mutations

Eight polymorphic sites of *NAT2* were analyzed.

PCR No. 1 Amplification of the whole exon of the *NAT2* gene (detection of the mutations 481C>T and 857G>A).

The whole exon (1211-bp fragments) of the *NAT2* gene was amplified. 50-80 ng of target DNA was amplified in a 50-µl reaction mix, which contained 5 µl PCR-buffer, 5 µl 2 mmol/l dNTPs, 1 µl of each of the primers P100 and P56, 1 U *Taq*-polymerase, 4.8 µl 25 mmol/l $MgCl_2$, and 33.2 µl H_2O. Thermocycling conditions were as follows: initial denaturation at 94°C for 2 min; 35 cycles of denaturation at 94°C for 30 s, annealing and elongation at 67°C for 90 s, and terminal extension at 72°C for 7 min. The PCR result was checked by electrophoresis in 1% agarose gel.

From the PCR-product which contained the 1211-bp fragment, two restriction digests were performed with the enzymes *Kpn*I and *Bam*HI for the detection of mutations 481C>T and 857G>A. The remaining PCR-product was diluted 1:10 by adding 162 µl H_2O until a final volume of 180 µl was reached.

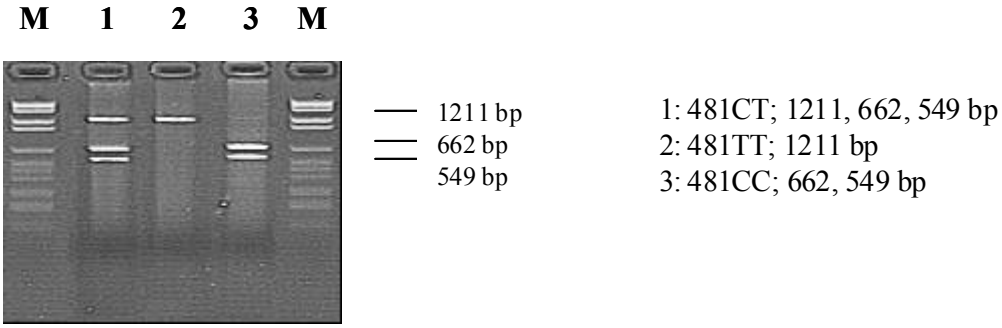


Figure 17: *Kpn*I digest of a 1211-bp fragment, containing positions for mutation 481C>T.

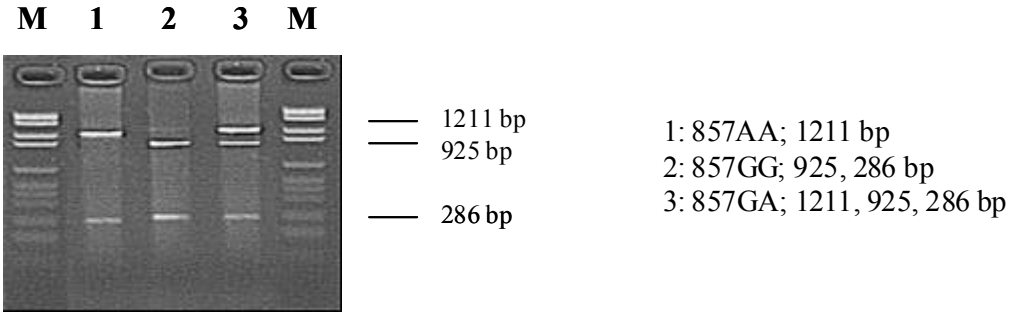


Figure 18: *Bam*HI digest of a 1211-bp fragment, containing positions for mutation 857A>G.

PCR No. 2 Detection of mutations 191G>A, 282C>T, 341T>C and 111T>C.

The nested PCR, using the primers P100 and P341N, was performed for the amplification of a 442-bp fragment, which contains the mutations 191G>A, 282C>T, 341T>C and the novel mutation 111T>C. One µl of the diluted PCR-product (P100/P56) was added to the mastermix. PCR-conditions were as follows: an initial denaturation at 94°C for 2 min, 14 cycles at 94°C for 0.5 min, at 67°C for 1.5 min and terminal elongation at 72°C for 7 min. For evaluation at nt position 111, 12.5 µl of PCR-product (P100/P341N) were incubated with the restriction endonuclease *Taq*I (10 U) at 65°C for 3-4 hours. Further mutations at positions 191 and 341 were investigated by overnight digestion of a 442-bp fragment with the endonucleases *Msp*I (50 U) and *Dde*I (5 U) at 37°C. For evaluation at nt position 282, the PCR-product was digested with *Fok*I (15 U) enzyme at 37°C for 2 hours.

The restriction-fragment lengths are presented in Table 15.

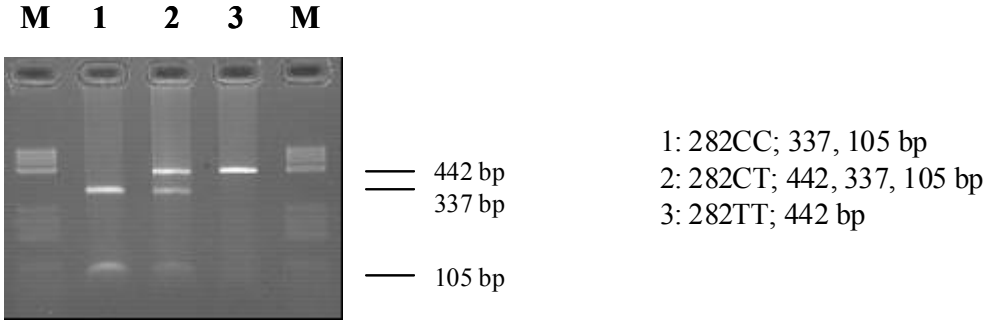


Figure 19: *Fok*I digest of a 442-bp fragment containing positions of the mutation 282C>T.

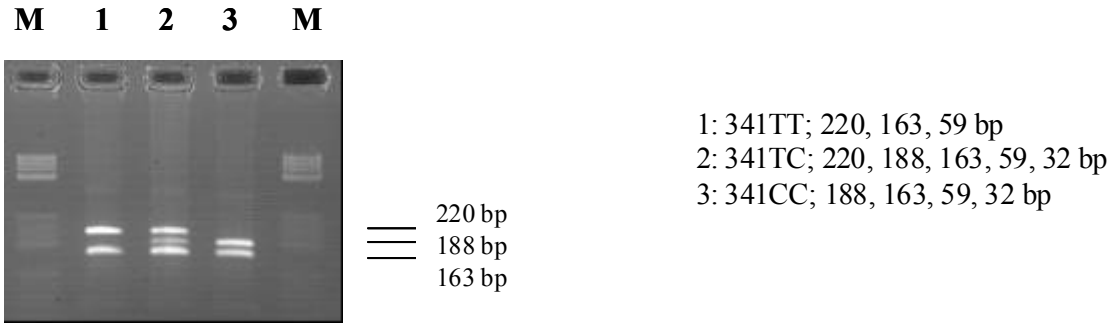


Figure 20: *Dde*I digest of a 442-bp fragment containing positions of the mutation 341T>C

PCR No. 3 Detection of mutations 590G>A and 803A>G.

For the detection of mutations at the positions 590 nt and 803 nt nested PCR with the primers P87 and P90 was performed from the initial 1211-bp amplificate (an initial denaturation at 94°C

for 2 min; 14 cycles at 94°C for 0.5 min, at 60°C for 1 min, at 72°C for 1 min; at 72°C for 7 min) and resulted in a 421-bp fragment. 1 µl of the PCR-product (P100/P56), which had been diluted 1:10, was added to the mastermix. 12.5 µl of the PCR-product were incubated with 10 *Taq*I U at 65°C for 3-4 hours for the detection of the mutation at position 590. Overnight digestion of 12.5 µl of the PCR-product was performed with 5 U *Dde*I at 37°C to detect the mutation at position 803.

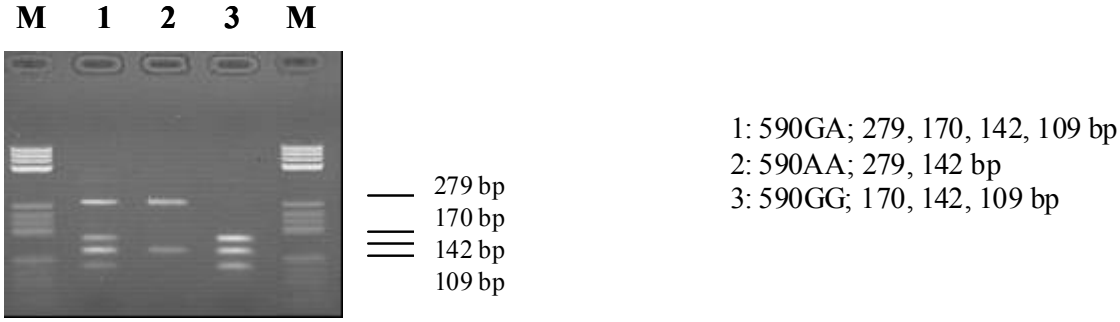


Figure 21: *Taq*I digest of a 421-bp fragment containing positions of the mutation 590G>A.

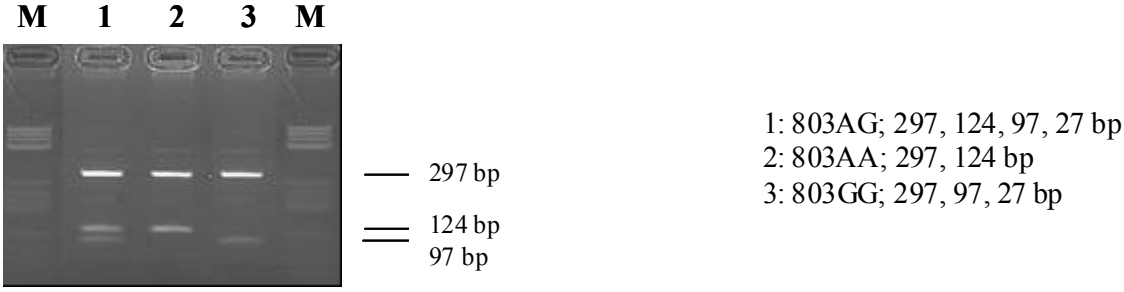


Figure 22: *Dde*I digest of a 421-bp fragment containing positions of the mutation 803A>G.

Table 14: Oligonucleotide-primers for amplification of coding regions of the *NAT2*-gene and amplification of small fragments containing possible mutations at positions 111, 191, 282, 341, 481, 590, 803 and 857.

Primer	Primer-length (nt)	Fragment-length (bp)	Sequence	Specificity
P100	-69- -48	1211	5'-GTCACACGAGGAAATCAAATGC	*NAT2*-gene (5')
P56	1142-1119	-	5'-GTTTTCTAGCATGAATCACTCTGC	*NAT2*-gene (3')
P341N	342-373	442	5'-ACCCAGCATCGACAATGTATTCC-TGCCCTCA	*Dde*I-site 341 nt (3')
P87	480-499	421	5'-CCTGGACCAAATCAGGAGAG	*Taq*I and *Dde*I-site 803 nt (5')
P90	900-879	-	5'-ACACAAGGGTTTATTTTGTTCC	(3')
P85	368-386	192	5'-CTGGGTCTGGAAGCTCCTC	(5')
P86	559-540	-	5'-GGTGTTTCTTCTTTGGCAGG	(3')

Table 15: Restriction endonucleases for recognition of *NAT2*-mutations.

Position	Transition	Restriction endonuclease	Recognition sequence	Preamplification with primers	Cleavage-site (nt) Wildtype* Mutation	Fragment-length (bp) Wildtype** Mutation
111	T>C	*Taq*I	T^CGA	P100/P341	- 111	442 262 180
191	G>A	*Msp*I	C^CGG	P100/P341	189 96	184 165 93 277 165
282	C>T	*Fok*I	GGATGnnnn nnnnn^nnnn	P100/P341	268 -	337 105 442
341	T>C	*Dde*I	C^TnAG	P100/P341	-2 153 -2 153 341	220 163 59 188 163 59 32
481	C>T	*Kpn*I	GGTAC^C	P100/P56	480 -	662 549 1211
590	G>A	*Taq*I	T^CGA	P87/P90	588 730 730	170 142 109 279 142
803	A>G	*Dde*I	C^TnAG	P87/P90	776 776 803	297 124 297 97 27
857	G>A	*BamH*I	G^GATCC	P100/P56	856 -	925 286 1211

* First line, wild-type cleavage-site; second line, mutant cleavage-site

** First line, wild-type fragment; second line, mutant fragment

***n – A, C, T or G

2.3.2.6 Identification of *NAT2* genotypes by continuous monitoring of fluorogenic hybridization probes

Sixth polymorphic sites (111, 341, 481, 590, 803 and 857) of the *NAT2* gene were analyzed, using rapid cycle DNA amplification with allele-specific fluorescent probes and melting curve analysis. The PCR primers for *NAT2* genotyping are presented in Table 17. The hybridization probes were so designed that their melting temperatures (T_m) were similar to or a little higher than the T_m of the primers. The one probe was labelled with fluorescein (X) at the 3'-end, the second hybridization probe was labelled with LightCycler Red 640 (LC-Red 640) at the 5'-end. The two probes recognized adjacent sequences with the shorter probe lying over the mutation site. The probes were separated by one to five bases. Fluorescein was used as the donor fluorophore and blocked extension from the probe during PCR. LC-Red, with its 3'-end phosphorylated to block extension, was used as the acceptor of the FRET process. The greater stability of the longer anchor probe led to a loss of fluorescence as soon as the shorter probe melted off the template.

Detection of the mutation 111T>C.

For the detection of the 111T>C polymorphism, a 421-bp fragment was amplified with the primers NAT2-26F and NAT2-425R and detected with the hybridization probes 111S and 111A (Table 17). A 20-µl PCR mix contained 2 µl PCR-buffer, 1 µl 2 mmol/l dNTPs, 1.5 µl 50 mmol/l $MgCl_2$, 0.4 µl of each of the primers, 0.2 µl of each of the hybridization probes, 1 µl DMSO, 0.6 µl 1 mg/ml BSA, 1 U *Taq*-polymerase, 12.5 µl H_2O, and 30 ng of genomic DNA.

Detection of the mutation 341T>C.

For the detection of the 341T>C polymorphism, a 421-bp fragment was amplified with the primers NAT2-26F and NAT2-425R and detected with the hybridization probes 341S and 341A. A 20-µl PCR mix contained 2 µl PCR-buffer, 1 µl 2 mmol/l dNTPs, 1.5 µl 50 mmol/l $MgCl_2$, 0.4 µl of each of the primers, 0.2 µl of each of the hybridization probes, 1 µl DMSO, 0.6 µl 1 mg/ml BSA, 1 U *Taq*-polymerase, 12.5 µl H_2O, and 30 ng of genomic DNA.

Detection of the mutation 481C>T.

PCR with the primers P85 and P86 resulted in a 192-bp fragment, and the 481C>T polymorphism was detected with the hybridization probes Sensor 5 and Anchor 5. A 20-µl PCR

mix contained 2 µl PCR-buffer, 1 µl 2 mmol/l dNTPs, 2.5 µl 50 mmol/l $MgCl_2$, 0.4 µl of each of the primers, 0.3 µl of each of the hybridization probes, 0.6 µl 1 mg/ml BSA, 1 U *Taq*-polymerase, 12.3 µl H_2O, and 30 ng of genomic DNA.

Detection of the mutation 590G>A.

For the detection of the 590G>A polymorphism, a 197-bp fragment was amplified with the primers P87 and P88 and detected with the hybridization probes Sensor 590 and Anchor 590. A 20-µl PCR mix contained 2 µl PCR-buffer, 1 µl 2 mmol/l dNTPs, 2 µl 50 mmol/l $MgCl_2$, 0.4 µl of each of the primers, 0.2 µl of each of the hybridization probes, 1 µl DMSO, 0.6 µl 1 mg/ml BSA, 1 U *Taq*-polymerase, 12 µl H_2O, and 30 ng of genomic DNA.

Detection of the mutation 803A>G.

A 198-bp fragment was amplified with the primers P89 and P90 and detected with the hybridization probes Sensor 803A and Anchor 803. A 20-µl PCR mix contained 2 µl PCR-buffer, 1 µl 2 mmol/l dNTPs, 2 µl 50 mmol/l $MgCl_2$, 0.4 µl of each of the primers, 0.2 µl of each of the hybridization probes, 1 µl DMSO, 0.6 µl 1 mg/ml BSA, 1 U *Taq*-polymerase, 12 µl H_2O, and 30 ng of genomic DNA.

Detection of the mutation 857G>A.

For the detection of the 857G>A polymorphism, a 198-bp fragment was amplified with the primers P89 and P90 and detected with the hybridization probes NAT2*857S and NAT2*857A. A 20-µl PCR mix contained 2 µl PCR-buffer, 1 µl 2 mmol/l dNTPs, 1 µl 50 mmol/l $MgCl_2$, 0.4 µl of each of the primers, 0.2 µl of each of the hybridization probes, 1 µl DMSO, 0.6 µl 1 mg/ml BSA, 1 U *Taq*-polymerase, 13 µl H_2O, and 30 ng of genomic DNA.

The temperature profiles used for the hybridization probes experiment are shown in Table 16.

Table 16: PCR conditions for the detection of mutations of *NAT2* using LightCycler.

Mutation	PCR Reaction					Melting curve, ramp rate, °C/s
	Initial denaturation	Denaturation	Annealing	Elongation	Number of cycles	
111T>C	95° 2 min	95° 3 s	56° 14 s	72° 20 s	60	0.1
341T>C	95° 2 min	95° 3 s	56° 14 s	72° 20 s	45	0.15
481C>T	95° 2 min	95° 3 s	60° 15 s	72° 15 s	42	0.15
590G>A	95° 2 min	95° 3 s	60° 12 s	72° 18 s	45	0.15
803A>G	95° 2 min	95° 3 s	60° 12 s	72° 18 s	58	0.05
857G>A	95° 2 min	95° 3 s	50° 12 s	72° 20 s	47	0.1

The differentiation of the PCR products was performed by the analysis of DNA melting curves in glass capillaries. First, DNA was denatured at 95°C, then maximal fluorescence was acquired by keeping the reaction at 40°C for 40 s. Melting curve data were generated by heating slowly to 95°C and collected continuously during that time. As soon as the shorter probe melted off the template, FRET no longer took place.

Table 17: Oligonucleotides used as PCR hybridization probes.

Position	Oligonucleotides		Sequence
111	Primers	NAT2-26F	5'- GACATTGAAGCATATTTTGAAAG
		NAT2-425R	5'- TCCTTCCCAGAAATTAATTCTAG
	Probes	111S	5'- LC Red 640-CATGTTAAGGTTCTCAAAGGGAACAG
		111A	5'- CCAACTCCATGGCTTGCCCACAAT-Flu
341	Primers	NAT2-26F	5'- GACATTGAAGCATATTTTGAAAG
		NAT2-425R	5'- TCCTTCCCAGAAATTAATTCTAG
	Probes	341S	5'- CAGGTGACCACTIICGGCAGGAATTACAT-Flu
		341A	5'- LC Red 640-TCGATGCTGGGTCTGGAAGCTCCTCCC
481	Primers	Primer 85	5'- CTGGGTCTGGAAGCTCCTC
		Primer 86	5'- GGTGTTTCTTCTTTGGCAGG
	Probes	Sensor 5	5'- LC Red 640-TCTGGTACCTGGACCAAATCAGGA
		Anchor 5	5'- GCATTTTCTGCTTGACAGAAGAGAGAGGA-Flu
590	Primers	Primer 87	5'- CCTGGACCAAATCAGGAGAG
		Primer 88	5'- GCAAGGAACAAAATGATGTGG
	Probes	Sensor 590	5'- TTGAACCTCAAACAATTGAA-Flu
		Anchor 590	5'- LC Red 640-TTTGAGTCTATGAATACATACCTGCAGACGTCT

803	Primers	Primer 89	5'- GTGGGCTTCATCCTCACCTA
		Primer 90	5'- ACACAAGGGTTTATTTTGTTCC
	Probes	Sensor 803A	5'- TTGAAGAAGTGCTAAAAATATTTAAGA-Flu
		Anchor 803	5'- LC Red 640-TTCCTTGGGGCGAAATCTCTGGC
857	Primers	Primer 89	5'- GTGGGCTTCATCCTCACCTA
		Primer 90	5'- ACACAAGGGTTTATTTTGTTCC
	Probes	NAT2*857S	5'- LC Red 640-CAAACCTGGTGATGGATCCCT
		NAT2*857A	5'- TTCCTTGGGGAGAAATCTCGTGC-Flu

2.4 Statistical analysis

All information about patients was collected in a databank. The databank was constructed with the Excel statistical program. Data were transferred to SPSS 10.0 and with this program all statistical tests were analyzed. All values given with 95% confidence interval were calculated with the χ^2 test. All results with a two-sided P value ≤ 0.05 were regarded as statistically significant. The comparison of the frequencies of single mutations, alleles and genotypes between different populations was performed with the statistical program Intercooled Stata 7.0. Expected genotype frequencies were calculated from allele frequencies with the Hardy-Weinberg equation ($1 = p^2 + 2pq + q^2$).

3 Results

3.1 Frequencies of *CYP1A1* point mutations and alleles

Seven point mutations of *CYP1A1* were detected. The data are presented in Table 18. The most frequent mutation was -4335G>A, which appeared in 25.8% of alleles. The frequencies of other point mutations were as follows: m1 (3801T>C) appeared in 9.8%, m2 (2455A>G) in 5%, m4 (2453C>A) in 2.5%, m6 (-3219C>T) in 6.0% and m7 (-3229G>A) in 2.9% of alleles. Mutation m3 (3205T>C) did not occur.

The frequencies of point mutations and alleles of *CYP1A1* are shown in Table 18.

Table 18: *CYP1A1* mutation and allele frequencies in the Russian sample, n=325.

CYP1A1 allele	Nucleotide-position (nt)							n	%	95% C. I.
	m1	m2	m3	m4	m5	m6	m7			
	3801	2455	3205	2453	-4335	-3219	-3229			
**1A(wt)*	T	A	T	C	G	C	G	364	56.0	52.1-59.9
**1B*	T	A	T	C	G	**T**	G	39	6.0	4.3-8.1
**1C*	T	A	T	C	G	C	**A**	0	0.0	0.0-0.6
**1D*	T	A	T	C	**A**	C	G	149	22.9	19.7-26.4
**1E*	T	A	T	C	**A**	C	**A**	19	2.9	1.8-4.5
**2A*	**C**	A	T	C	G	C	G	30	4.6	3.1-6.5
**2B*	**C**	**G**	T	C	G	C	G	33	5.1	3.5-7.1
**4*	T	A	T	**A**	G	C	G	16	2.5	1.4-4.0
n	64	33	0	16	168	39	19	650		
%	9.8	5.0	0.0	2.5	25.8	6.0	2.9		100.0	
95% C. I.										
Min.	7.7	3.5	0.0	1.4	22.5	4.3	1.8			
Max.	12.4	7.1	0.6	4.0	29.4	8.1	4.5			

3.2 Genotype frequencies of *CYP1A1*

Table 19: Genotypes of *CYP1A1* which occurred in this study

Genotype	n	%	95% C. I.	Expected, %*
**1A/*1A*	106	32.7	24.5-38.0	31.5
**1A/*1B*	21	6.6	4.0-9.7	6.8
**1A/*1D*	81	25.0	20.3-30.0	25.7
**1A/*1E*	11	3.4	1.7-6.0	3.3
**1A/*2A*	17	5.3	3.1-8.2	5.3
**1A/*2B*	16	5.0	2.8-7.9	5.8
**1A/*4*	6	1.8	0.7-3.9	2.9
**1B/*1D*	10	3.0	1.5-5.6	2.8
**1B/*1E*	2	0.6	0.07-2.2	0.4
**1B/*2A*	1	0.3	0.01-1.7	0.7
**1B/*2B*	2	0.6	0.07-2.2	0.7
**1B/*4*	3	0.9	0.2-2.7	0.4
**1D/*1D*	14	4.3	2.4-7.1	5.3
**1D/*1E*	6	1.8	0.7-3.9	1.4
**1D/*2A*	8	2.5	1.1-5.0	2.2
**1D/*2B*	12	3.8	1.9-6.4	2.4
**1D/*4*	4	1.2	0.3-3.1	1.2
**2A/*2B*	2	0.6	0.07-2.2	0.6
**2A/*4*	2	0.6	0.07-2.2	0.3
**2B/*4*	1	0.3	0.01-1.7	0.3
Sum	325	100.0		

* Expected genotype frequencies are calculated from the frequencies of the single alleles (Table 18) according to the Hardy-Weinberg law.

3.3 Allele frequencies of *CYP2D6*

We have investigated the functionally important alleles which occur most frequently among Caucasians: *CYP2D6*1, *3, *4, *5* (gene deletion), **6*, Asiatic allele **10*, and allele duplications **1x2*, **2x2*. The data are presented in Table 20. The frequency of the gene-duplication of the highly active *CYP2D6* wild-type allele **1x2* was 1.7% (0.8-3.2), that of the duplication of the slightly active allele **2x2* was 1.0% (0.2-3.0). The duplication of the deficient allele **4* was not found in the Russian sample. We did not differentiate alleles with slightly decreased activity, such as **2, *9* and **10*.

Table 20: The frequencies of *CYP2D6* alleles in the Russian sample, n=290.

Allele	Nucleotide position (nt)						n	%	95 % C. I.
	188	1749	1795	1934	2637	4268			
**1*	C	G	T	G	A	G	411	70.8	67.0-74.5
**3*	C	G	T	G	**Del**	G	6	1.0	0.4-2.2
**4*	**T**	**C**/G	T	**A**	A	**C**	105	18.2	15.1-21.5
**6*	C	G	**Del**	G	A	G	7	1.2	0.5-2.5
**10*	**T**	**C**	T	G	A	**C**	24	4.2	2.7-6.1
**1x2*	(*CYP2D6*1* Duplication)						10	1.7	0.8-3.2
**2x2*	(*CYP2D6*2* Duplication)						3	0.5	0.1-1.5
**4x2*	(*CYP2D6*4* Duplication)						0	0.0	0.0-0.6
**5*	(*CYP2D6* Deletion)						14	2.4	1.3-4.0
Total							580	100.0	

3.4 Frequencies of *CYP2D6* genotypes

Genotype frequencies and distribution of groups with different metabolic activity in the Russian sample are shown in Table 21.

Table 21: Genotype frequencies of *CYP2D6*.

Genotype	n	%	95% C. I.	Expected %*
3 active genes (UM/EM)	10	3.4	1.7-6.3	2.6
**1x2/*1*	10	3.4	1.7-6.3	2.6
2 active genes (EM/EM, EM/IM, IM/IM, UM/PM)	168	57.9	52.0-63.7	56.5
**1/*1*	148	51.0	45.1-56.9	50.1
**2x2/*4*	3	1.0	0.2-3.0	0.2
**1/*10*	16	5.5	3.2-8.8	5.9
**10/*10*	1	0.3	0.0-1.9	0.2
1 active gene (EM/PM, IM/PM)	95	32.8	27.4-38.5	34.2
**1/*3*	4	1.4	0.4-3.5	1.5
**1/*4*	70	24.1	19.3-29.5	25.8
**1/*5*	10	3.4	1.7-6.3	3.6
**1/*6*	5	1.7	0.6-4.0	1.8
**4/*10*	6	2.1	0.8-4.5	1.5
0 active genes (PM/PM)	17	5.9	3.5-9.2	5.0
**3/*4*	2	0.7	0.1-2.5	0.4
**4/*4*	9	3.1	1.4-5.8	3.3
**4/*5*	4	1.4	0.4-3.5	0.9
**4/*6*	2	0.7	0.1-2.5	0.4
Sum	290	100.0		

* Expected genotype frequencies are calculated from the frequencies of the single alleles (Table 20) according to the Hardy-Weinberg law.

3.5 Frequencies of *CYP2D6* genotypes, according to gender

The sample of Russian study participants consisted of 152 men and 138 women. The frequencies of UMs and EMs were slightly higher in women – 3.7% of UM and 60.3% of EM in comparison to 3.3% UM and 55.9% EM in men (n. s.). The data found in different gender groups is presented in Table 22.

Table 22: Frequencies of *CYP2D6* genotypes in 152 men and 138 women in a Russian sample.

CYP2D6 genotype	Men		Women	
	n	%	n	%
UM	5	3.3	5	3.7
EM	85	55.9	83	60.3
IM	53	34.9	42	30.6
PM	9	5.9	8	5.4
n	152	100.0	138	100.0

3.6 Frequencies of *CYP2D6* genotypes, according to age

The age of participants ranged from 14 to 77 years. They were assigned to three different age groups. Ninety-one individuals were under 30 years and 150 were older than 40 years. The distributions of *CYP2D6* genotypes among the three different age groups are presented in Table 23.

Table 23: Frequencies of *CYP2D6* genotypes in different age groups in a Russian sample.

CYP2D6 genotype	Age group					
	< 30		30-40		> 40	
	n	%	n	%	n	%
UM	2	2.2	2	4.1	6	4.0
EM	54	59.3	23	46.9	91	60.4
IM	30	33.0	22	44.9	43	28.9
PM	5	5.5	2	4.1	10	6.5
n	91	100.0	49	100.0	150	100.0

3.7 Frequencies of *NAT2* point mutations and alleles

In this study seven out of eight tested point mutations could be allocated to eight different allelic variants. The 341C>T transition was the most frequent mutation in Russians, it appeared in 42.6%. The 111T>C mutation occurred in only 0.3% of individuals. We found neither the specific Black-African 191G>A mutation nor unknown haplotypes. The frequencies of the investigated point mutations are presented in Table 24. There were two alleles which led to rapid-acetylation activity (*NAT2*4* and **12*), and six alleles (*NAT2*5A, *5B, *5C, *6A, *6D* and **7B*) which were associated with slow acetylation activity.

Table 24: Frequencies of *NAT2* alleles and point mutations in 325 healthy Russian volunteers and patients without any known malignant disease.

NAT2 allele	Nucleotide position (nt)								Pheno-type	n	%	95% C. I.
	111	191	282	341	481	590	803	857				
**4(wt)*	T	G	C	T	C	G	A	G	Rapid	145	22.3	19.2-25.7
**5A*	T	G	C	**C**	**T**	G	A	G	Slow	15	2.3	1.3-3.8
**5B*	T	G	C	**C**	**T**	G	**G**	G	Slow	242	37.3	33.5-41.1
**5C*	T	G	C	**C**	C	G	**G**	G	Slow	17	2.6	1.5-4.2
**6A*	T	G	**T**	T	C	**A**	A	G	Slow	208	32.0	28.4-35.7
**6D*	**C**	G	**T**	T	C	**A**	A	G	Slow	2	0.3	0.04-1.1
**7B*	T	G	**T**	T	C	G	A	**A**	Slow	19	2.9	1.8-4.5
**12A*	T	G	C	T	C	G	**G**	G	Rapid	4	0.6	0.2-1.6
**14A*	T	**A**	C	T	C	G	A	G	?	0	0.0	0.0-0.6
**14B*	T	**A**	**T**	T	C	G	A	G	?	0	0.0	0.0-0.6
n	2	0	223	277	256	204	264	19				
%	0.2	0.0	17.9	22.2	20.6	16.4	21.2	1.5				
95 % C. I.												
Min.	0.02	0.0	15.8	20.0	18.4	14.4	19.0	0.9				
Max.	0.6	0.3	20.2	24.7	22.9	18.6	23.6	2.4				

3.8 Frequencies of *NAT2* genotypes

Table 25 shows the distribution of *NAT2* genotypes among Russians. One hundred and thirty one subjects had a genotype associated with fast acetylation (40.3%). Eighteen individuals (5.5%) had the homozygous rapid-acetylator genotype (*NAT2*4/*4* and *NAT2*4/*12*), and 112 subjects were heterozygous for the alleles *NAT2*4* or **12A* which encode a rapid phenotype. The number of slow acetylators, coded by alleles *NAT2*5A, *5B, *5C, *6A, *6D,* and **7B,* amounted to 59.7%. The most common genotypes leading to slow acetylation were *NAT*5B/*6A* (25.3%) and **5B/*5B* (13.6%). The genotype combinations **4/*12A, *5B/*12A, *5A/*7B*, and **7B/*7B* were found only once each. The genotype frequencies were in good agreement with the expected values as calculated with the Hardy-Weinberg equilibrium from the frequencies of single alleles (Table 24).

Table 25: Distribution of *NAT2* genotypes among 325 Russian individuals.

NAT2 genotype	N	%	95% C. I.	Expected %*
**4/*4*	17	5.3	3.1-8.2	4.9
**4/*5A*	2	0.6	0.07-2.2	1.0
**4/*5B*	52	16.0	12.2-20.5	16.6
**4/*5C*	3	0.9	0.2-2.7	1.2
**4/*6A*	47	14.5	10.8-18.8	14.3
**4/*7B*	6	1.8	0.7-3.9	1.3
**4/*12A*	1	0.3	0.01-1.7	0.3
**5B/*12A*	1	0.3	0.01-1.7	0.4
**6A/*12A*	2	0.6	0.07-2.2	0.4
Not found	-			
Sum rapid	131	40.3	34.9-45.9	40.4
**5A/*5B*	6	1.8	0.7-3.9	1.7
**5A/*6A*	6	1.8	0.7-3.9	1.5
**5A/*7B*	1	0.3	0.01-1.7	0.1
**5B/*5B*	44	13.6	10.0-17.6	13.9
**5B/*5C*	7	2.2	0.9-4.4	1.9
**5B/*6A*	82	25.3	20.6-30.3	23.9
**5B/*7B*	6	1.8	0.7-3.9	2.1
**5C/*6A*	5	1.5	0.5-3.6	1.7
**5C/*7B*	2	0.6	0.07-2.2	0.2
**6A/*6A*	30	9.3	6.3-12.9	10.2

**6A/*6D*	2	0.6	0.07-2.2	0.4
**6A/*7B*	2	0.6	0.07-2.2	1.9
**7B/*7B*	1	0.3	0.01-1.7	0.1
Not found	-			
Sum slow	194	59.7	54.1-65.1	59.6
Total	325	100.0		

* Expected genotype frequencies are calculated from the frequencies of the single alleles (Table 24) according to the Hardy-Weinberg law.

3.9 Identification of *N*-acetyltransferase 2 genotypes by continuous monitoring of fluorogenic hybridization probes

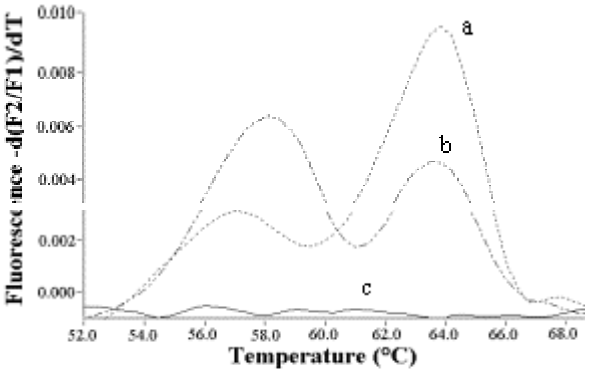
127 samples were checked for sixth mutations at the positions 111, 341, 481, 590, 803 and 857 of *NAT2* which allow detecting all known genotypes of *NAT2* in Caucasians. We used a new technique which combines a microvolume fluorimeter with a rapid temperature cycler (LightCycler). Homogenous curves were observed for wild-type and mutant alleles, whereas heterogeneous curves resulted from the simultaneous presence of both alleles.

The melting temperatures of wild-type alleles and mutant alleles are present in Table 26. When hybridization probes formed a perfect match with the target, this resulted in a comparatively stable complex and, consequently, in a high T_m. As expected, a lower T_m was observed in the case of a mismatch. Samples containing both alleles (heterozygous) displayed 2 melting peaks at exactly the same temperatures as the respective homozygous samples. Negative controls without added template did not show any signal.

Table 26: Melting temperature of wild-type and mutant alleles of *NAT2*.

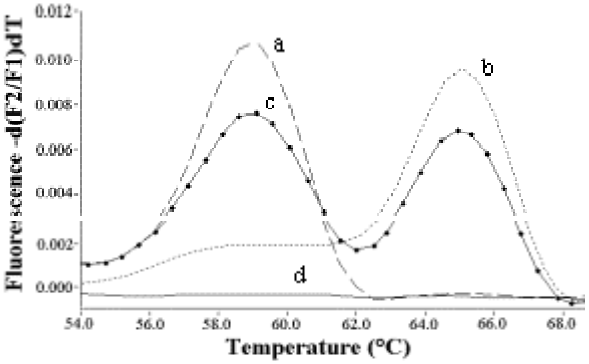
Position	T_m (wild type)	T_m (mutation)
111	64.0°C	58.0°C
341	59.0°C	65.0°C
481	61.5°C	68.0°C
590	47.0°C	54.7°C
803	49.5°C	46.5°C
857	61.5°C	57.5°C

The melting peaks derived from these data are shown below.



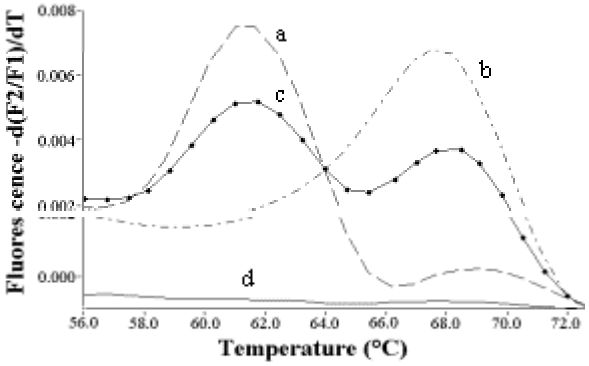
a – 111TT
b – 111TC
c – negative control

Figure 23: Detection of the mutation 111T>C.



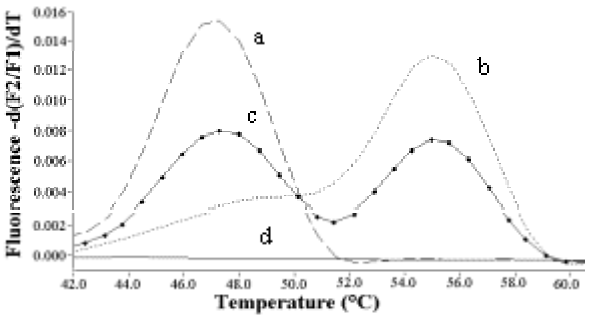
a – 341TT
b – 341CC
c – 341TC
d – negative control

Figure 24: Detection of the mutation 341T>C.



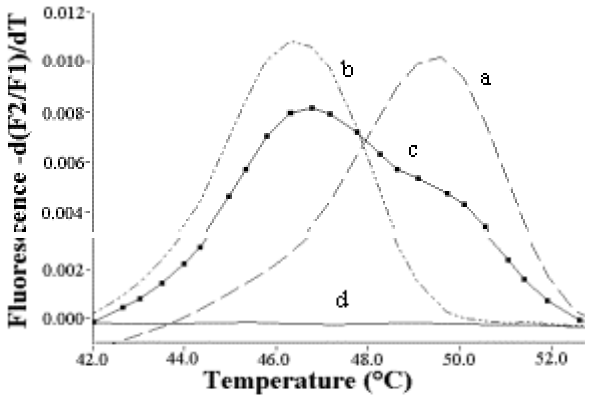
a – 481CC
b – 481TT
c – 481CT
d – negative control

Figure 25: Detection of the mutation 481C>T.



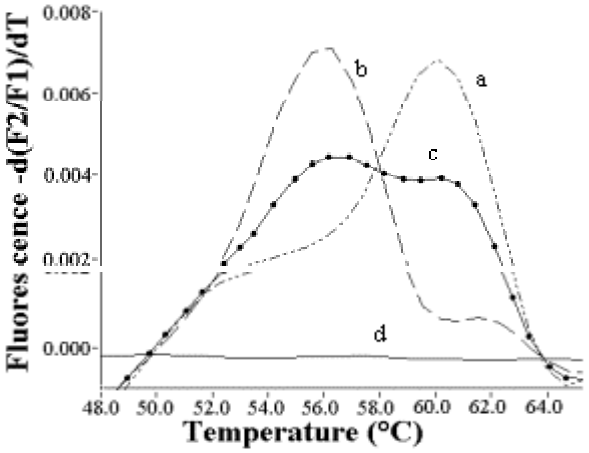
a – 590GG
b – 590AA
c – 590GA
d – negative control

Figure 26: Detection of the mutation 590G>A.



a – 803AA
b – 803GG
c – 803AG
d – negative control

Figure 27: Detection of the mutation 803A>G.



a – 857GG
b – 857AA
c – 857GA
d – negative control

Figure 28: Detection of the mutation 857G>A.

4 Discussion

Population frequencies of many pharmacogenetics traits have been shown to depend on ethnic specificity. This was the first study which investigated allele frequency distributions of drug and xenobiotic-metabolizing enzymes in a Russian sample. The number of investigated samples was large enough to find even rare alleles of polymorphic genes.

4.1 Interethnic variability of enzymes of phases I and II

In order to avoid the accumulation of harmful xenobiotics in cells, living organisms have developed ways for their elimination. Multiple xenobiotic metabolizing enzymes with different and partially overlapping catalytic properties play a key role in the elimination process. These enzymes are encoded by superfamilies of genes, the evolution of which has enabled different species to survive in different habitats and to take advantage of diets containing certain harmful xenobiotics. As a result of this evolutionary process, species have achieved capacities for metabolizing which are appropriate for their survival, but which may differ considerably from those of other species. This evolutionary process may also explain the interethnic and interindividual variability of drug metabolism in humans.

4.1.1 Genotype frequencies of *CYP1A1*

The frequency of the known seven cytochrome P450 1A1 (*CYP1A1*) gene mutations was investigated in 325 Russians. The mutations m1 (6235T>C) and m2 (4889A>C) were not significantly different from the German population: m1: 9.8% of alleles and 7.7% (p=0.1), m2: 5.0% of alleles and 2.7% (p=0.06) in Russians and Germans, respectively, the frequency of m4 (2453C>A) did differ in the two populations – 2.5% and 3%, respectively (German data from Cascorbi *et al*. (Cas96c)). M2 was found to be exclusively linked with the m1 forming allele *CYP1A1*2B*. *CYP1A1* mutations tended to be more frequent in the Russian sample than in other Caucasian groups, but were rarer than in a Turkish population (m1 – 18.1% (p=0.0004), m2 – 8.9% (p=0.04), m4 – 5.7% (p=0.007), according to Aynacioglu *et al*. (Ayn98)) or in the Far East (m1 appears with the frequency of 33.2% in Japanese, p<0.0001 (Nak91)). The frequencies of the *CYP1A1* high-activity alleles, *CYP1A1*2A* and *CYP1A1*2B*, were 4.6% (3.1%-6.5%) and 5.1% (3.5%-7.1%), respectively. The frequency of the *CYP1A1*2A* allele was comparable with the frequency in the Polish population, namely 4.5% (Mro97). The frequencies of m5, m6 and m7 mutations in different populations are not available up to now.

4.1.2 Genotype frequencies of *CYP2D6*

The frequencies of CYP2D6 genotypes vary between populations. The deficient allele **4* is the allele with the highest ethnic variability. The virtual absence of the common "white" **4* allele in East Asian populations explains the very low prevalence of the PM trait in these people. *CYP2D6*5* occurs in Orientals as frequently as in Caucasians and Africans and accounts for the few poor metabolizers in Orientals (Joh91). The allele frequency of *CYP2D6*10* is high (50%) in the Oriental population and low (5%) in Caucasians (Arm94, Joh94). Genotyping of African populations (Mas93, Mas96, Akl96) revealed the absence of the *CYP2D6*3* allele and the low prevalence of *CYP2D6*4* thus explaining the low prevalence of poor metabolizers in these populations. Possibly, allele **4* appeared in Caucasoids only after their separation from the Asian/Amerindian group about 35.000 years ago. The allele **3* appears to be rare except in Whites, whereas the deletion allele **5* occurs with comparable frequency in most populations. The allele **17* was found exclusively in African populations (Mas96) and was postulated to be the basis of the diminished debrisoquine hydroxylase activity in these populations.

In this study, only those population frequencies of allelic variants of the highly polymorphic enzyme CYP2D6 have been determined which are functionally most important. We did not investigate the frequency of allele **2* (with the exception of the allele **2* duplication), which has been shown to have only slightly decreased activity in comparison with allele **1* (Sac97). In the case of allele duplications the determination of the duplicated allele (**2x2* or **4x2*) is clinically important in a correct prediction of the metabolic capacity to avoid misclassification of **4* duplication as ultrarapid allele. The frequencies of alleles **9* and **7* were relatively low in Caucasians in previous studies (Sac97), so we did not include these and more rare alleles, such as **8, *11, *12, *14, *15* in this study.

The frequencies of the defective alleles **3, *4, *5* and **6* found in our study were comparable with those found in earlier studies of Caucasians (Sac97, Lea98). The frequency of allele duplications in Russians (2.2%) was not significantly different from other Caucasians: duplications were found in 2.0% of Germans (Sac97), in 1.9% of Frenchmen (Leg98), and in 1.0% of the Swedish population (Dah95). In contrast, a high frequency of duplicated alleles was described in black Ethiopians and Saudi Arabians (Table 27).

Table 27: The frequency of deficient and duplicated alleles of *CYP2D6* in different populations.

Population	n	Allele frequency (%)					Reference
		**3*	**4*	**5*	**6*	**MxN*	
Japanese	256	nf*	0.8	4.1	0.5	nd**	Chi99
Koreans	152	nd	nd	nd	nd	0.3	Roh96
Chinese	113	nd	nd	nd	nd	1.3	Joh94
Germans	589	2.0	21.0	2.0	0.9	2.0	Sac97
Polish	145	2.1	23.1	0.7	nd	nd	Gaw99
French	265	nd	nd	nd	nd	1.9	Leg98
Russians	290	1.0	18.2	2.4	1.2	2.2	This study
South-Spanish	217	nd	12.0	3.9	nd	3.5	Agu95
North-Spanish	147	nd	20.0	3.4	nd	5.1	Bern97
Turkish	404	nf	11.0	1.0	nf	6.0	Ayn99
West-Africans	326	nf	6.3	6.0	nf	1.6	Gri99
Saudi Arabians	101	nd	3.5	1.0	nd	10.0	McL97
Black Ethiopians	122	nd	nd	nd	nd	16.0	Akl96
Black Tanzanians	106	nf	6.3	6.0	nf	nd	Wen99

* not found

** not detected

4.1.3 Genotype frequencies of *NAT2*

The prevalence of slow acetylator genotypes in the Russian sample was found to be similar to other Caucasian populations. The frequencies of slow acetylator genotypes vary considerably among ethnic groups. Genotyping data of the common *NAT2* alleles helps us to understand the molecular history of the acetylation polymorphism (Table 28). Two groups can be distinguished by the prevalence of the mutations 341T>C/481C>T (*NAT2*5A, B, C*) and 857G>A (*NAT2*7A, B*): in Caucasians and Africans the frequencies of alleles **5* are high and the frequencies of alleles **7* are low whereas it is just opposite in the Japanese, Chinese, and in other Asian populations. Thus, the difference in the prevalence of the slow acetylator phenotype between Caucasians/Africans and Asian populations is mostly the result of the low prevalence of the *NAT2*5* alleles in Asians. Asian populations show high frequencies of the wild-type allele *NAT2*4* (44 to 79%) in comparison with other ethnic groups (6 to 24%). The frequency in

Hispanics is intermediate between the Caucasians/Africans and the Asian groups. The data is consistent with the theory that the acetylation polymorphisms have an ancient and common African origin, which dates back to the period before the spreading of human populations in the paleolithic. The practical absence of the *NAT2*12* and *NAT2*14* alleles in Caucasians and the high frequency of 191G>A in Africans suggests that these mutations appeared after the African/Non-African split about 90 000 years ago.

Table 28: Comparison of allele frequencies (%) of *NAT2* between different ethnic groups.

Population	n	**4*	**5A*	**5B*	**5C*	**6A*	**6D*	**7B*	**12A*	**14B*	Reference
African	117	9.4	1.3	28.2	6.4	24.4	nd*	1.3	12.0	7.3	Del96
German	1088	23.4	2.5	40.9	2.6	28.4	nd	2.1	0.1	0.1	Cas95
Turkish	303	23.1	1.3	35.6	4.8	30.5	nd	4.5	0.2	nf**	Ayn97
Polish	248	22.0	5.2	33.1	6.0	30.0	nd	3.4	0.2	nf	Mro96
Russian	325	22.3	2.3	37.3	2.6	32.0	0.3	2.9	0.6	nf	This study
Japanese	145	68.6		2.4§		19.3	nd	9.7	nd	nf	Oku97
Chinese	187	51.0		7.5§		32.0	nd	10.0	nd	nd	Lee98
Hispanic	137	41.6		31.4§		16.8	nd	nd	nd	nd	Mar98

*- not detected, **- not found

§- alleles of the group **5* were not differentiated

4.2 Individual pharmacotherapy adjusted to genotype

Metabolism of many drugs influences their pharmacological and toxicological effects. One of the major causes of interindividual variation of drug effects is genetic variation of drug metabolism. Polymorphisms, which cause decreased, increased, or lacking enzyme expression or activity by multiple molecular mechanisms, are generated by mutations in the genes for drug-metabolizing enzymes.

The type and prevalence of allelic variants present in a population will influence the pharmacological and toxicological effects of drugs, toxins and carcinogens and lead to interindividual and interethnic differences.

The observation that individuals who are genetically deficient in a particular P450 enzyme are poor metabolizers of one or several drugs illustrates a very important principle; namely that the

rate of elimination of drugs can be largely determined by a single P450 enzyme. This observation seems to contradict the fact that P450 enzymes have broad and overlapping substrate specificities. The explanation of this evident paradox lies in the fact that although more than one human P450 enzyme can catalyze the biotransformation of xenobiotics, their affinities may be markedly different. Consequently, xenobiotic biotransformation in vivo, where only low substrate concentrations are usually achieved, is often determined by the P450 enzyme with the highest affinity for the xenobiotic. For example, the *N*-demethylation of diazepam and the 5-hydroxylation of omeprazole are both catalyzed by two P450 enzymes, namely CYP2C19 and CYP3A4. However, these reactions are catalyzed by CYP3A4 with such low affinity that the *N*-demethylation of diazepam and the 5-hydroxylation of omeprazole in vivo appear to be dominated by CYP2C19 (Kat94). Similarly, tolbutamide is metabolized by both CYP2C9 (70%) and CYP2C19 (30%) (Ino97, Wes00, Ven98, McG00) and ibuprofen by CYP2C9 (90%) and CYP2C19 (10%) (Leem93, Hamm97). The most important drug metabolizing P450 enzymes are presented in Figure 29.

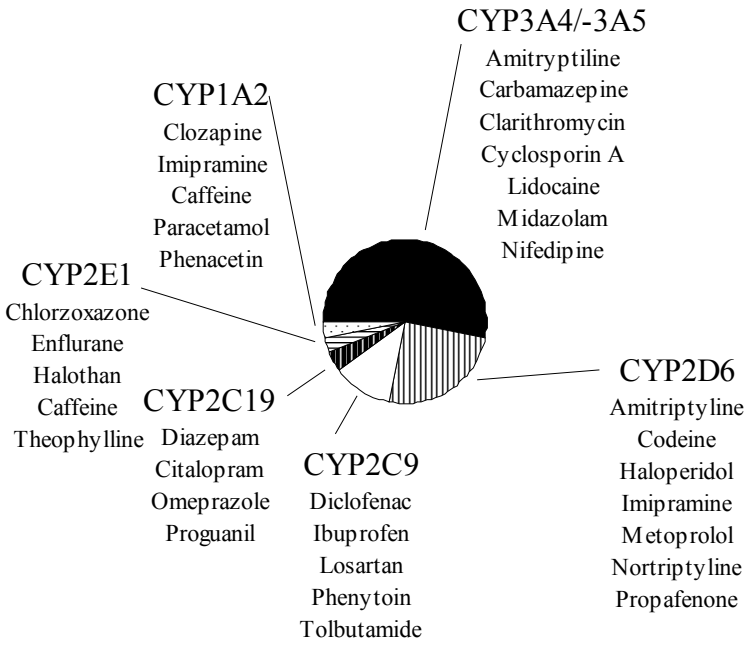


Figure 29: The role of different P450 enzymes in metabolism of drugs (Ben95).

Sometimes two or more P450 enzymes contribute equally to the metabolism of a single compound. In the metabolism of propranolol for example, CYP2D6 oxidizes the aromatic ring to give 4-hydroxypropranolol, CYP1A2 mediates ethoxyresorufine *O*-deethylation, and CYP2C19 oxidizes the isopropranolamine side chain. Consequently, changes in either CYP2D6 or

CYP1A2 or CYP2C19 do not markedly affect the disposition of propranolol. Three human P450 enzymes, CYP1A2, CYP2E1, and CYP3A4, can convert the commonly used analgesic, acetaminophen, to its hepatotoxic metabolite, *N*-acetylbenzoquinoneimine. It is also possible that a single P450 enzyme catalyzes two or more metabolic pathways of the same drug.

4.2.1 Genetic susceptibility to adverse drug reactions

Possible risk factors for drug toxicity or inefficacy are the age of the patient, concomitant diseases such as liver or renal diseases, lifestyle habits such as smoking or alcohol consumption, or drug-drug interactions. Hereditary factors that affect the kinetic and dynamic parameters of various drugs are of great importance in the determination of the individual risk. Genetic polymorphisms in the genes for drug metabolizing enzymes, drug transporters and drug receptors are related to individual variability in the efficacy and toxicity of drugs (Mey91, Ing99, Evan99, Ros00).

Genetic polymorphisms of these enzymes give rise to distinct subgroups in the population that differ in their ability to perform certain drug biotransformation reactions. In specific sub-populations undergoing treatment, this can lead to a variety of consequences, such as therapeutic failure, adverse effects and toxicity, which are often difficult to foresee. The incidence of serious and fatal adverse drug reactions has been found to be extremely high among patients; adverse drug reactions caused about 100 000 deaths in the USA in 1994 (Laz98).

Genetic analysis of drug metabolizing enzymes is clinically important for determining the appropriate dosage of certain drugs; thus it prevents therapeutic failures, adverse effects and toxicity. Knowing the genotype of a patient might help improve drug efficacy and reduce toxicity - by selecting the right drug for the right patient. When mutations result in null alleles (no catalytic activity), drugs may reach toxic levels if given in standard doses. Some typical adverse drug reactions caused by CYP450 polymorphisms are presented in Table 29.

Table 29: Cytochrome P450 enzyme gene polymorphism and possible adverse drug reactions.

P450 enzyme	Frequency of polymorphism	Drug	Adverse reaction		Reference
			In slow metabolizers	In fast metabolizers	
CYP1A2	5-10% deficient	Antipsychotics		Tardive dyskinesia	Bas00
CYP2C9	2-3% (homozygous)	Warfarin	Haemorrhage		Ait99
		Tolbutamide	Hypoglycaemia		Mine98
	15-20% (heterozygous)	Phenytoin	Phenytoin toxicity		Kid99
		Losartan	Decreased antihypertensive effect		Mine98
CYP2C19	2-4% (Caucasians) 10-25% (Asians) (homozygous)	Diazepam	Prolonged sedation		Mey00
CYP2D6	5-10% (poor metabolizers)	Antiarrhythmics	Arrhythmias		Mey00
		Beta-Blockers	Bradycardia		
	1-10% (ultrarapid metabolizers)	Tricyclic antidepressants	Toxicity	Inefficacy	Mey00
		Opioids	Inefficacy of codeine as analgesic	Narcotic side-effects, dependence	Mey00

In patients with a defective CYP variant, genotyping could help select drugs that do not depend upon the defective CYP for excretion. Because there are various CYP isoforms, individuals who poorly metabolize one drug may have normal metabolism of another.

Average dose recommendations for drugs with genetically polymorphic elimination and deactivation may be considered as a compromise to achieve sufficiently safe and effective treatment in all patients, including PMs and EMs. Thus, these standard dosages are probably lower than optimal for the EM group, and higher than optimal for the PM group.

It is certainly very useful to know the patient's genotype; nevertheless, drug metabolism is complex and might be complicated by various factors: (1) multiple mutations and alleles with varying effects (> 50 alleles for *CYP2D6* alone), (2) greatly varying allele frequencies for each CYP isoform in different populations, (3) effects of heterozygocity on drug metabolism, (4) possible interaction of one drug with more than one CYP isoform, (5) unexpected drug-drug interactions in a patient carrying null alleles in one CYP isoform through inhibition of other CYP isoforms.

1.1.4 Impact of the *CYP2D6* genotype on drug treatment

The clinical impact of polymorphisms of CYP2D6 is a subject of great interest with special attention paid to cardiovascular and neuroactive drugs, because many CYP2D6 substrates belong to these classes of drugs. CYP2D6 enzyme activity ranges from relatively low to ultra-rapid. Allele frequencies of *CYP2D6* which lead to poor metabolizer status or extensive metabolizer status for many drugs mainly metabolized by CYP2D6 range from 1% to 10% in different populations. Generally, poor metabolizers and extensive metabolizers differ between 2- and 5-fold in their capacities to metabolize CYP2D6 substrates; hence PMs obtain the same steady-state serum level as EMs with only 20-50% of the doses (Bros93).

The *CYP2D6* genotype has been shown to predict the clearance of antidepressants (Spi97, Car96, Ham96) as well as that of neuroleptics (Jer96). Numerous case reports and clinical studies have demonstrated that the CYP2D6 polymorphisms could lead to a higher propensity to develop adverse reactions at conventional doses. It has been shown that individuals who lack functional *CYP2D6* genes metabolize certain CYP2D6 substrates, particularly antidepressants and neuroleptics, at a lower than normal rate. Thus, PMs may be treated with unnecessarily high doses of neuroleptics, resulting in a higher number of side effects which may worsen the patient's compliance with the treatment (Sha93). Antipsychotics have a very narrow therapeutic window. Plasma concentrations above the therapeutic level only increase the risk of extrapyramidal side effects without further increasing the antipsychotic activity (Han81). Adverse effects due to elevated drug plasma levels would be expected to occur more frequently when the drug clearance depends entirely on CYP2D6. Sometimes decreased drug effects or therapeutic failure occur at normal antidepressant doses in ultrarapid metabolizers, as it has been described in the rare subjects with 13 *CYP2D6* copies on one allele. They synthesized substantially higher amounts of metabolites of nortriptyline than those carrying fewer active *CYP2D6* genes (Dal98). Antiarrhythmics have pro-arrhythmic effects in 10% to 20% of patients, and high concentration of these drugs in plasma has been identified as a predisposing factor. As there is a correlation between antiarrhythmic activity and plasma concentration for most antiarrhythmics, drug dosage should be adjusted to achieve and maintain steady-state concentrations in the therapeutic range. A patient with cardiac failure, renal and/or liver failure who also happens to be a PM will be at a high risk of developing side effects. Vomiting and arrhythmias during treatment with propafenone (Bot94) and mexiletine (Lle93) have been seen to occur only in PMs and were attributed to elevated plasma drug concentration as a consequence of lower metabolic rate.

On the one hand, PMs are at increased risk of accumulating drugs and suffering from drug-related toxicity, on the other hand they might experience therapeutic failure with prodrugs that need metabolization to achieve pharmacological activity. A lack of CYP2D6 enzyme activity should result in reduced efficacy when prodrugs requiring activation by CYP2D6 are used. An absence of the analgetic effect of codeine in PMs due to their inability to form morphine has been described by Poulsen *et al*. (Pou96). At the same time, severe abdominal pain, a typical adverse effect of morphine, was observed in ultra-rapid metabolizers treated with codeine because of extensive formation of morphine (Dal97).

Cytochrome P450 2D6 is a drug-metabolizing enzyme with a limited metabolizing capacity even in EMs. Due to its saturation during the first pass, it metabolizes drugs such as propafenone and imipramine in a dose-dependent manner (Sid87, Bros88). CYP2D6 is also the site of a number of drug interactions (Wag87). Substrates with a very high affinity to the enzyme such as propafenone bind strongly to it and inhibit the metabolism of compounds with lower affinity. These drug interactions occur in extensive as well as in poor metabolizers and may lead to clinically important effects, as has been documented for the combination of certain neuroleptics with antidepressants (Nel80).

The intermediate active *CYP2D6*10* allele occurs rather frequently in Oriental populations. It results in a certain metabolic insufficiency, the clinical consequences of which are reflected in the literature: the lower doses of neuroleptics used in Asians compared with Caucasians (Lin83), the higher plasma haloperidol concentration and a higher prolactin response to haloperidol (Lin88), the impaired formation of morphine from codeine (Tse96), and the disposition of the Beta-adrenoreceptor antagonist propranolol (Lay95). Guided by their practical observations of drug efficacy and side effects, clinicians in Oriental populations have been prescribing antipsychotics at lower doses than physicians in Caucasian populations; it is now postulated that the differences in drug metabolism resulted in different prescribing habits (Lin91).

Since the therapeutic efficacy and the adverse effects of many drugs depend on CYP2D6 activity, genotyping of *CYP2D6* may become a part of routine laboratory examination. The increasing knowledge of the CYP2D6 phenotype and genotype status in different populations could be used to design genotype-based dose-finding studies. Some steps have already been taken in this direction. Kirchheiner *et al*. (Kir01) suggested preliminary genotype-based dose recommendations for antidepressants which are metabolized by CYP2D6 and CYP2C19.

1.1.5 Inhibition of cytochrome P450

In addition to predicting the likelihood of some individuals being poor metabolizers due to a genetic deficiency in P450 expression, information on which human P450 enzyme metabolizes a drug can help predict or explain drug interactions (Pec93).

Inhibitory drug interactions generally fall into three categories. The first involves competition between two drugs which are metabolized by the same P450 enzyme. For example, omeprazole and diazepam are both metabolized by CYP2C19. When the two drugs are administrated simultaneously, omeprazole decreases the plasma clearance of diazepam and prolongs its plasma half-life. The inhibition of diazepam metabolism by omeprazole is presumed to involve competition for metabolism by CYP2C19 because no such inhibition occurs in individuals who, for genetic reasons, lack this polymorphically expressed P450 enzyme. The second inhibitory drug interaction is also competitive in nature, but the inhibitor is not a substrate of the affected P450 enzyme. The inhibition of dextromethorphan biotransformation by quinidine is a good example of this type of drug interaction. Dextromethorphan is *O*-demethylated by CYP2D6, and the clearance of dextromethorphan is impaired in individuals lacking this polymorphically expressed enzyme. The clearance of dextromethorphan is similarly impaired when this antitussive agent is taken with quinidine, a potent inhibitor of CYP2D6. The third type of drug interaction results from noncompetitive inhibition of cytochrome P450, and it often involves so-called mechanism-based inhibition (as in the case of grapefruit juice, which inhibits the presystemic elimination of a number of drugs and increases their bioavailability), or suicide inactivation of cytochrome P450 (Hal94), when the metabolites of some compounds bind to the heme and destruct it. The inhibition of terfenadine metabolism by macrolide antibiotics appears to be an example of this type of drug interaction. CYP3A4 converts macrolide antibiotics to a metabolite that binds so tightly (but noncovalently) to the heme moiety of CYP3A4 that it is not released from the enzyme's active site. The noncompetitive inhibition of a P450 enzyme by a mechanism-based inhibitor can completely block the metabolism of a drug. As the fatal interaction between macrolide antibiotics and terfenadine indicates, noncompetitive inhibition of cytochrome P450 can have serious consequences. Numerous compounds are activated by cytochrome P450 to metabolites that bind covalently to the heme moiety or to the surrounding protein. These compounds, known as suicide inactivators, include various halogenated alkanes (CCl_4), halogenated alkenes (vinyl chloride, trichloroethylene), allylic compounds (allylisopropylacetamide and secobarbital), and acetylenic compounds (ethinylestradiol and ethynylprogesterone, gestodene). Ethinyl derivatives of various P450 substrates have been

synthesized as potential selective mechanism-based inhibitors of individual P450 enzymes. For example, polycyclic aromatic hydrocarbons are preferred substrates of CYP1A1, and this enzyme can be activated by various ethinyl derivats of naphthalene and pyrene.

4.2.2 Induction of cytochrome P450

In contrast to inhibitors, inducers of cytochrome P450 increase the rate of xenobiotic biotransformation (Con67, Con82, Bat92). Clinically important consequences of P450 enzyme induction include the enhanced biotransformation of cyclosporin, warfarin, and contraceptive steroids by inducing the CYP3A4 and CYP2C enzymes, and enhanced activation of acetaminophen to its hepatotoxic metabolite, *N*-acetylbenzo-quinoneimine, by the CYP2E1 inducers, ethanol and isoniazid, and possibly by CYP3A4 enzyme inducers. As an underlying cause of serious adverse effects, P450 induction is generally less important than P450 inhibition, because the latter can cause a rapid and large increase in the blood level of a drug, which can cause toxic effects and symptoms of drug overdose. In contrast, cytochrome P450 induction lowers blood levels, which compromises the therapeutic goal of drug therapy but does not cause an exaggerated response to the drug. An exception to this rule is the potentiating effect of alcohol and isoniazid on acetaminophen hepatotoxicity, which is in part because of cytochrome P450 induction.

4.2.3 Clinically relevant polymorphisms of *NAT2*

The polymorphism in *N*-acetylation leads to the interindividual differences in the metabolism of drugs and xenobiotics which have a primary aromatic amine or a hydrazine structure. Genetically determined differences in NAT2 activity may play an important role in the treatment with NAT2 substrates. The frequency of side effects after use of drugs that are acetylated greatly differs in slow and rapid acetylators (Roo92): isoniazid hepatotoxicity (Tim77), sulfasalazine-induced agranulocytosis (Wad00) and side effects during the treatment with co-trimoxazol (Zie98) were more frequent in slow acetylators. A significant association between isoniazid-induced and rifampicin-induced hepatotoxicity and the *NAT2* genotype was show by Ohno *et al*. (Ohn00). The relative risk, compared with that of the rapid type, was 4.0 (95% C. I., 1.94%-6.06%) for the intermediate type and 28.0 (95% C. I., 26.0%-30.0%) for the slow type. Table 30 presents the most common adverse effects of drugs metabolized by acetylation.

Table 30: Adverse effects of drugs – substrates of arylamine *N*-acetyltransferase 2 in relationship to the acetylator phenotype (according to Evans (Eva92)).

Drug	Indication	Possible adverse effects	Phenotype
Aminoglutethimide	Breast cancer	Confusion, tiredness, vertigo, sickness, diarrhea, myelosuppression	slow
Amonafide	Breast cancer	Myelosuppression, confusion, tinnitus, vertigo, sickness	slow
Amrinone	Heart failure	Arrhythmia, gastrointestinal symptoms thrombozytopenia	slow rapid
Dapsone	Lepra	Allergic skin reactions, methemoglobinemia, myelosuppression	slow
Isoniazid	Tuberculosis	Peripheral neuropathy hepatotoxicity	slow rapid
Hydralazine	Hypertension	Lupoid syndrome	slow
Procainamide	Tachyarrhythmia	Lupoid syndrome, gastrointestinal symptoms, hypotension	slow
Sulfalene	Malaria prophylaxis	Allergic reactions, gastrointestinal symptoms, changes in the blood count, lupoid syndrome	slow
Sulfasalazine	Rheumatoid arthritis, Crohn´s disease	Vertigo, headache, reticulocytosis, gastrointestinal symptoms	slow
Sulfamethazine	Infection, phenotyping	Allergic symptoms	slow

NAT2 genotyping prior to medication may help discover patients who have a great risk of developing adverse side effects which are dependent on differences in the metabolism.

4.2.4 Pharmacogenetic studies and the practice of medicine

Hereditary polymorphisms in drug metabolizing enzymes and drug transporters determine the majority of adverse drug effects and drug ineffectiveness. It is to be expected that the increasing genetic knowledge will have considerable impact on disease management and healthcare in the future. It is already possible to identify sensitive individuals and to recommend individualized

genotype-dependent drug treatment, especially for drugs with a narrow therapeutic window. This allows to prevent adverse reactions already before drug treatment and thus substantially reduce the costs of therapy and hospitalization.

The knowledge about the mechanism of drug action, the identification of new drug targets, and the understanding of genetic factors that affect drug response may allow to design drugs that are specifically targeted towards particular subgroups of the population or that avoid genetic variability in therapeutic response.

4.3 The role of polymorphisms of drug metabolizing enzymes in the occurrence of some kinds of cancer

Genetic differences in the regulation, expression and activity of phase I and phase II genes, encoding drug metabolizing enzymes might be crucial factors in defining cancer susceptibility, as well as in determining the toxic or carcinogenic potential of drugs and other environmental pollutants.

Several epidemiological studies in cancer patients have shown that individual susceptibility to cancer might partly depend on the genetically determined high or low activity of certain enzymes. This association may be based on interindividual variation in the metabolism of carcinogens that are either detoxified, or metabolically activated to ultimate carcinogens. Cytochrome P450 2D6, glutathione *S*-transferase M, and *N*-acetyltransferase 2 have been primarily studied because of their well-known inheritable polymorphism and the availability of routine methods to determine an individual's phenotypic expression.

Research work has focused on two groups of classic environmental carcinogens: polycyclic aromatic hydrocarbons (PAH), generated from the combustion of fossil fuels, and aromatic amines, which are for example present in cigarette smoke. Both PAH and aromatic amines are major etiologic factors in lung, bladder, and possibly in breast cancers. Variations in the expression of the metabolic genes, such as the cytochrome P450, glutathione *S*-transferase (*GST*), and *N*-acetyltransferase (*NAT*) genes, strongly influence the individual biological response to carcinogens. The carcinogenic residues which bound to DNA or proteins, and which are known as adducts, provide both a fingerprint of exposure and an indicator of procarcinogenic DNA damage. In general, more PAH-DNA adducts are formed in smokers or in persons who are exposed to PAH at the workplace or in the ambient air. However, various studies have shown a 30- to 50-fold interindividual difference in carcinogen-DNA binding under equivalent conditions of exposure (Per96).

A number of drug metabolizing enzyme superfamilies, including cytochrome P450 dependent monooxygenases, and other enzymes and receptor proteins have probably evolved as an adaptive response to environmental stimuli. Most drug metabolizing enzymes are primarily detoxifying enzymes, but CYP1A1, glutathione *S*-transferase T1 and sulfotransferase lead to an activation in the metabolism of polycyclic aromatic hydrocarbons, vicinal dihaloalkanes and methylated PAH oxides by catalyzing the generation of electrophilic metabolites. It is reasonable to assume that mutants or variant alleles which lead to an alteration in the activity of one of these enzymes would result in an altered susceptibility to chemically induced diseases, such as cancer and certain occupational diseases.

The identification of susceptible populations could lead to the elucidation of mechanisms of disease and help to design the preventive strategies that are of the greatest benefit.

4.3.1 Genetic polymorphism of *CYP1A1* and cancer susceptibility

The possible relationship between cytochrome P450 1A1, smoking, and lung cancer reveals the complex epidemiological background of environmentally induced disease. Some studies suggest an association between a particular *CYP1A1* allele and smoking-induced lung cancer. However, the frequency of alleles varies markedly among different ethnic groups, so that extrapolation between populations is not justified.

Allelic variant forms of *CYP1A1* have been extensively studied in association with lung cancer susceptibility in a case-control epidemiology designs. The mutation m1 (3801T>C) was found to be overrepresented among lung cancer patients in Japan (Nak91). Studies in Caucasians could not confirm this finding (Tef91, Hir92b, Dra94), perhaps due to the lower allele frequency of 7.3% (Dra94) compared to 33.2% in Japanese (Nak91). The A>G transition at nucleotide 2455 (m2) is rare in Caucasians but occurs in about 20% of Japanese (Kaw93). The mutant enzyme showed enhanced activity. M2 was overrepresented in a Japanese study (Nak91) and also in a German study of lung cancer patients (Dra94), whereas only a trend was observed in a Finnish study (Hir92a). The mutation m4 (2453C>A) was shown not to represent a susceptibility factor for lung cancer (Cas96c). Hence, individuals with high *CYP1A1* gene inducibility may be more susceptible to the carcinogens in tobacco smoke and they may be more likely to develop tobacco-related lung cancer. Genes other than *CYP1A1* may also influence the incidence of cancer induced by smoking. Interactions of the recognized polymorphisms of *CYP1A1* with other susceptibility polymorphisms in other genes (for example glutathione *S*-transferase M1

(*GSTM1*)) may be more relevant to lung carcinogenesis than any other polymorphism alone. The combination of phase I and phase II genotypes (example *CYP1A1* Val/Val and *GSTM1* null) has suggested possible susceptibility genotypes in the Japanese population, where odds ratios for lung cancer cases who displayed combined susceptible genotypes have ranged as high as 27 (Nak93, Kih95), these combinations of genotypes are currently being explored in non-Asian populations.

Genetic variation in receptors that are instrumental in the toxicokinetics of carcinogens can strongly influence cancer risk. Individuals with the high-affinity dioxin-binding aromatic hydrocarbon (Ah) receptor are likely to be at greater risk from dioxin and PAH, because by binding to the receptor, these chemicals up-regulate *CYP1A1*, *CYP1A2*, and other genes, thereby stimulating their own metabolism (Neb91).

4.3.2 The influence of the *CYP2D6* genotype on cancer susceptibility

Studies of associations between the *CYP2D6* polymorphism and susceptibility to specific diseases, particularly lung cancer, have produced conflicting results. The debrisoquine PM phenotype has been reported to be under-represented in smokers with lung carcinoma in comparison to noncancer controls (Aye84, Roo88, Law89, Cap90, Agu94, Bou96). A meta-analysis of the data published until 1992 reported an overall odds ratio of 2.3 of lung cancer patients displaying an intermediate or extensive metabolizer phenotype (Amo92). A Finnish study reported an odds ratio of 6.4 ($p=0.05$) in the EM phenotype in comparison to controls (Hir93). In a Spanish study, the *CYP2D6*9* has been reported to be six times more frequent in lung cancer cases than in healthy controls (Agu94). However, a French group has reported no statistically significant relationship between this genotype and lung cancer status (Stu95), and there are several case-control studies that have found no relationship between debrisoquine phenotype prevalence and lung cancer (Ran95, Ben91, Duc91, Stu95, Shaw95). One confounder appears to be the race; probably, the association seen in Caucasians cannot be extrapolated to Afro-Americans (Ing92) or Asians (Bert92). In summary, the data addressing the relationships between CYP2D6 genotype, phenotype and lung cancer susceptibility remains conflicting and inconclusive.

Brockmöller *et al*. (Bro96) investigated the role of different *CYP2D6* alleles as susceptibility factors or protective factors in bladder cancer etiology; however, no association was found.

It has been reported that persons with EM genotype of CYP2D6 and slow acetylator *N*-acetyltransferase 2 (*NAT2*) genotype were at higher risk of developing hepatocellular carcinoma (Agu96).

4.3.3 The role of *N*-acetyltransferase 2 in the predisposition to aromatic and heterocyclic amine-induced carcinogenesis

A number of aromatic amines such as 4-amino-biphenyl and heterocyclic amines such as 2-amino-1-methyl-6-phenylimidazo pyridine are present in cigarette smoke (Man91) and in well-done meat (Lay95). Aromatic and heterocyclic amine carcinogens require metabolic activation for DNA mutation and initiation of carcinogenesis. Following *N*-oxidation, the *N*-hydroxy-aromatic and *N*-hydroxy-heterocyclic amines are further activated by *N*-acetyltransferases to their ultimate carcinogenic forms (Hei88, Hei95). Both NAT1 and NAT2 catalyze the metabolic activation of aromatic and heterocyclic amine carcinogens (Min92, Hei93, Hei95). Thus, genetic polymorphisms in *NAT1* and *NAT2* may affect the metabolic activation of aromatic and heterocyclic amine carcinogens and modify cancer risk. Epidemiological studies investigating the role of *NAT1* and *NAT2* polymorphisms in colorectal cancer illustrate the complexity of the etiologic background. The current mechanistic hypothesis suggests that rapid NAT1 and NAT2 acetylators should more readily activate *N*-hydroxy-heterocyclic amine carcinogens to their ultimate carcinogenic forms; therefore, these individuals are predisposed to colorectal cancer. However, human populations are genetically heterogeneous and exposures to heterocyclic amines are difficult to estimate. Therefore, it is not surprising that the results of studies which tried to verify the hypothesis are not consistent: while several investigations (Lan86, Ile87, Gil98) found an association between the rapid NAT2 acetylator phenotype and colorectal cancer, other studies (Lad91, Rod93, Shi94, Pro95, Spu95, Hub97) did not. Five studies (Lan94, Rob96, Wel97, Che98, Kam99) found associations between the rapid NAT2 acetylator phenotype and colorectal cancer primarily in individuals who consumed well-done meat, and, presumably were exposed to larger quantities of heterocyclic amine carcinogens (Sko95). One study found that the association was confined to homozygous rapid acetylators (Gil98), a finding also observed for lung cancers (Cas96b) and laryngeal (Hen99) cancers. Bell *et al*. (Bel95) found an association between the *NAT1*10* allele and colorectal cancer; the risk was highest in NAT2 rapid acetylators. Another study also showed a higher risk for colorectal cancer in individuals who consumed well-done meat and possessed both the *NAT1*10* allele and a rapid acetylator *NAT2* genotype (Che98). *NAT1*10* and *NAT2*4* (the most common rapid acetylator *NAT2* allele) are in a linkage disequilibrium (Sme98, Hen99) which may be a factor in the association of the *NAT1*10* allele with colorectal cancer. However, two studies reported a lack of association between the *NAT1*10* allele and colorectal cancer (Pro96, Lin88).

Recently investigations have examined the possible relationship between *NAT1* and *NAT2* acetylation polymorphisms and breast cancer risk. Zheng *et al*. (Zhe99) observed an association of the *NAT1*11* allele with breast cancer, particularly in women who smoked or consumed their meat very well done. In another study, an association between the rapid/intermediate NAT2 acetylator phenotype and breast cancer was found in women who consumed well-done meat (Dei00). Many other investigations, which have been published recently, suggest that the acetylation polymorphism is a factor in genetic predisposition to cancers of different types especially when linked to aromatic and heterocyclic amine carcinogen exposures.

4.3.4 Cancer susceptibility related to ethnicity or race

Epidemiological data shows that ethnic and racial groups differ significantly in terms of cancer incidence and mortality rates (Zah95). Cancer incidence rates of esophageal cancer in black Americans are approximately three times higher than in white Americans; incidence rates of multiple myeloma, liver, cervical, and stomach cancer are twice as high; and those of cancers of the oral cavity and pharynx, larynx, lung, prostate, and pancreas are 50% higher. The incidence of chronic lymphocytic leukemia, multiple myeloma, and premenopausal breast cancer is also higher in black Americans. In contrast, white Americans have higher incidence rates of melanoma, leukemia, lymphoma, and cancers of the endometrium, thyroid, bladder (in males), ovary, testis, and brain, as well as postmenopausal breast cancer (ACS97). In Hispanics cancer rates are generally lower than in white or black Americans, but rates differ substantially among Hispanics of different race (Per96).

Among the biologic factors that might contribute to the higher cancer risks in certain ethnic or racial groups are variations in the prevalence of genetic traits affecting carcinogen metabolism and DNA repair. The stronger association between the serum concentration of the DDT metabolite DDE and breast cancer in black women relative to white women may reflect genetic differences affecting the induction of the enzyme-mediated estrogen metabolism by chlorinated hydrocarbons (Mil95). The *GSTM1* null genotype occurs less frequently in Blacks (35%) than in Whites (49%) (Bel93). The NAT2 slow acetylator phenotype occurs in about 59% of Whites (Cas95), in 55% of Blacks (Del96), and in 14% of Asians (Yu94), these frequencies are consistent with the respective ethnic or racial differences in bladder cancer rates.

Racial or ethnic variation in cancer risk may reflect differences in environmental exposure or socioeconomic and demographic factors as well as in hereditary biologic susceptibility. The rise in breast cancer rates experienced by the descendants of Asian immigrants to the United States

provides strong evidence that environmental factors affect cancer patterns. After several generations, the rates in Asian Americans are the same as those of the U.S. white population (Zah95, Mil95). Within the United States, substantial ethnic or racial variations in the extent of environmental exposure to certain pollutants have also been recorded (Per96).

5 Summary

The basic principle of drug and xenobiotic metabolism in the body is to make them more water soluble and thus more readily excreted in the urine. Drug and xenobiotic transformation reactions are divided into two groups, namely phase I and phase II. Various drug metabolizing enzymes of phases I and II are subject to genetic polymorphism. The activity of these enzymes can vary from zero to high. Genetic polymorphisms are known to contribute considerably to interindividual variations in the metabolism of numerous drugs and xenobiotics. The phase I enzymes of the cytochrome P450 family (CYP450) are the most important enzymes in drug and xenobiotics metabolism. Among the human CYP isoforms, CYP2D6 seems to be one of the most relevant. It catalyzes the metabolism of nearly 25% of all clinically important drugs, such as antiarrhythmic drugs, tricyclic antidepressants, beta-blockers and others. The CYP1A1 enzyme is responsible for the oxidative metabolism of toxicants such as dioxin and benzo(*a*)pyrene. The phase II metabolizing enzyme arylamine *N*-acetyltransferase 2 (NAT2) plays an important role in the biotransformation of a number of aromatic and heterocyclic amines and hydrazines, which are contained in various drugs and in environment. Genetic polymorphisms of drug metabolizing enzymes are responsible for altered enzyme level and activity and associated with altered risk of adverse drug reactions and some cancers.

The allele frequency of genes, encoding phase I and phase II enzymes has been described for a number of populations and revealed a wide ethnical variation. However, this are no data available about the polymorphisms of drug metabolizing enzymes in the Russian population, which is the biggest Slavic one. That is why we investigated the genotypes of some of the genes, encoding drug and xenobiotic metabolizing enzymes of phases I and II and compared our results with the corresponding data from other populations. In this way we wanted to classify whether Russians are genetically similar to other Caucasian groups or whether there is some Asian influence.

In this study we investigated 325 individuals of Russian origin, who were healthy volunteers or patients without malignant diseases. Our study included the complete investigation of two

enzymes of phase I, CYP1A1 and CYP2D6, and one phase II enzyme, NAT2. We determined all known mutations of *CYP1A1*: m1 (3801T>C), m2 (2455A>G), m3 (3205T>C), m4 (2453C>A), m5 (-4335G>A), and the novel m6 (-3219C>T) and m7 (-3229G>A) mutations. We screened for the functionally important alleles **1,*3, *4, *5, *6, *10* of the *CYP2D6* gene and for gene duplications, which is sufficient to predict a metabolizing phenotype in 95-99% of individuals (Sac97). We checked on the most common *NAT2* point mutations: 191G>A, 282C>T, 341T>C, 481C>T, 590G>A, 803A>G, 857G>A, and the novel 111T>C mutation.

In addition to conventional PCR-RFLP genotyping of *NAT2* polymorphisms, 127 samples were analyzed using a novel LightCycler method. For this method we optimized a number of reactions with hybridization probes. This assay provides a basis for the rapid detection of the *NAT2* genotype in large-scale studies or routine genotyping in the laboratory.

The mutations m1 (3801T>C), m2 (2455A>G), m4 (2453C>A), m5 (-4335G>A), m6 (-3219C>T), and m7 (-3229G>A) of *CYP1A1* occurred in 9.8% (95% confidence interval, 7.7%-12.4%), 5.0% (95% C. I., 3.5%-7.1%), 2.5% (1.4%-4.0%), 25.8% (22.5%-29.4%), 6.0% (4.3%-8.1%), and 2.9% (1.8%-4.5%) of alleles, respectively. The frequencies of m1, m2 and m4 were similar to those in other European groups, but differed statistically significantly from those in a Turkish sample ($p=0.0004$ for m1 mutation, $p=0.04$ for m2 mutation and $p=0.007$ for m4 mutation (Ayn98)) and in a Japanese sample ($p<0.0001$ for m1 mutation (Nak91)). Mutation m2 was found to be strictly linked with m1, forming allele *CYP1A1*2B*. We did not find the m3 mutation, which has only been detected in Africans up to now. The frequencies of the *CYP1A1* high-activity alleles, *CYP1A1*2A* and *CYP1A1*2B*, were 4.6% (3.1%-6.5%) and 5.1% (3.5%-7.1%), respectively.

5.9% (3.5%-9.2%) of all subjects were CYP2D6 poor metabolizers, whereas 3.4% (1.7%-6.3%) were identified as ultra-rapid metabolizers (*CYP2D6*1x1/*1*). The mutation spectrum in *CYP2D6* and the proportion of intermediate and extensive metabolizers in Russians (34.2%, and 57.9%, respectively) were similar to those of other Caucasians.

Genotyping eight different single nucleotide polymorphisms in the *NAT2* gene provided a genotype associated with slow acetylation in 59.7% (54.1%-65.1%) of individuals, 34.7% (29.6%-40.2%) of participants carried at least one allele encoding rapid acetylation, and 5.6% (3.3%-8.6%) were homozygous for the rapid-acetylation allele (wild-type allele **4* or mutant allele **12A*). The frequencies of the point mutations, alleles and genotypes of *NAT2* of the Russian sample were similar to those of other Caucasians. As in other European groups, the

prevalence of slow acetylators in Russians appeared to be due to the high frequency of **5* alleles. The Black-specific 191G>A was not detected in our sample.

As the fluorescence genotyping of six polymorphisms of *NAT2*, using LightCycler, correlated in all samples with previously validated genotyping by restriction enzyme digestion (PCR-RFLP), we can recommend this method as a reliable, fast and easy way of *NAT2* genotyping.

The complete overview of allele distribution of the most important drug and xenobiotic metabolizing enzymes among Russians shows that the allele frequency is similar to that of other Caucasians. We did not identify any Asian influence in our Russian sample. No specific African genetic traits were detected in our sample. Therefore it may be expected that drug side effects and efficacy problems due to an individual's genetic background are similar compared to those in other European populations.

This comprehensive set of data should give a basis for clinical studies on drug disposition in the Russian population and further investigations of the role of polymorphic enzymes in the development of different kinds of cancer. Individual therapy recommendations for drugs metabolized by polymorphic drug metabolizing enzymes should be introduced into clinical practice to adjust the individual doses to genotype, determine the appropriate dosage of certain drugs and thus prevent therapeutic failures, adverse effects and toxicity.

6 Zusammenfassung

Die Umwandlung in wasserlösliche Verbindungen, die renal ausgeschieden werden können, ist ein grundlegendes Prinzip im Abbau von Fremdstoffen. Hierbei unterscheidet man Phase-I- und Phase-II-Reaktionen. Die Aktivität vieler Phase-I- und Phase-II-Enzyme ist genetisch beeinflusst und kann weniger und stärker ausgeprägt sein. Genetische Polymorphismen sind für die starken interindividuellen Unterschiede im Metabolismus von Fremdstoffen verantwortlich. Die Phase-I-Enzyme der Cytochrom-P450-Familie sind für den Abbau der meisten Arzneimittel sowie toxischen Fremdstoffen essentiell. Von den CYP-Isoenzymen ist CYP2D6 am Abbau von nahezu 25% aller klinisch eingesetzten Arzneistoffe (z.B. Antiarrhythmika, Antidepressiva, Beta-Blocker) beteiligt. Das CYP1A1-Enzym abbaut Toxine wie Dioxin und Benzo(a)pyren. Das Phase-II-Enzym Arylamin-*N*-Acetyltransferase 2 (NAT2) spielt eine wichtige Rolle in der Biotransformation von aromatischen und heterozyklischen Aminen und Hydrazinen, die als Medikamente und in der Umwelt vorkommen. Genetische Polymorphismen der Enzyme des Fremdstoffmetabolismus sind für eine veränderte Expression und Aktivität der betroffenen

Genprodukte verantwortlich und können das Krebsrisiko und das Risiko für Arzneimittelnebenwirkungen beeinflussen.

Die Häufigkeitsverteilungen der Allele der Gene, die Phase-I- und Phase-II-Enzyme kodieren, sind für viele Populationen bekannt; sie zeigen eine große interethnische Varianz. Da die Polymorphismen dieser Enzyme bisher jedoch noch nicht in der größten slawischen Volksgruppe, der russischen, untersucht wurden, haben wir die Häufigkeitsverteilungen verschiedener Genvarianten analysiert und unsere Ergebnisse mit den entsprechenden Daten anderer Populationen verglichen mit dem Ziel, Ähnlichkeiten oder Unterschiede zu anderen kaukasischen bzw. zu asiatischen Bevölkerungsgruppen aufzuzeigen.

An der vorliegenden Studie nahm eine Gruppe von 325 Personen russischer Abstammung teil - gesunde Probanden bzw. Patienten, die nicht an einer malignen Erkrankung litten. Die Polymorphismen von zwei Enzymen der Phase I, CYP1A1 und CYP2D6, und von einem Enzym der Phase II, NAT2, wurden komplett untersucht. Wir bestimmten alle bekannten Mutationen von *CYP1A1* - m1 (3801 T>C), m2 (2455A>G), m3 (3205T>C), m4 (2453C>A), m5 (-4335G>A) - sowie die erst vor kurzem entdeckten Varianten m6 (-3219C>T) und m7 (-3229G>A). Die funktionell bedeutsamsten Allele des *CYP2D6*-Gens (**1,*3, *4, *5, *6, *10*) sowie die Genduplikationen, die für die Erkennung des Phänotyps in 95-99% der Fälle ausreichen (Sac97), wurden bestimmt. Wir überprüften die häufigsten *NAT2* Punktmutationen - 191G>A, 282C>T, 341T>C, 481C>T, 590G>A, 803A>G, 857G>A - und die kürzlich entdeckte 111T>C Mutation.

Zusätzlich zur konventionellen PCR-RFLP-Genotypisierung des *NAT2* Polymorphismus analysierten wir 127 Proben mit der LightCycler-Hyprobe Methode. Real-time-PCR-Verfahren, wie die von uns eingesetzte LightCycler-Technologie, ermöglichen eine zuverlässige Genotypisierung in kürzester Zeit. Dies vereinfacht die Durchführung pharmakogenetischer Studien oder Routineuntersuchungen im Labor erheblich.

Die Häufigkeiten der genetischen Varianten von *CYP1A1* waren: m1 (3801T>C) - 9,8% (95% Vertrauensbereich, 7,7%-12,4%), m2 (2455A>G) - 5,0% (95% VB, 3,5%-7,1%), m4 (2453C>A) - 2,5% (1,4%-4,0%), m5 (-4335G>A) - 25,8% (22,5%-29,4%), m6 (-3219C>T) - 6,0% (4,3%-8,1%), und m7 (-3229G>A) - 2,9% (1,8%-4,5%). Die Häufigkeitsverteilung von m1, m2 und m4 ähnelt anderen Europäern, unterscheidet sich aber signifikant von den Ergebnissen einer türkischen ($p=0,0004$ für Mutation m1, $p=0,04$ für Mutation m2 und $p=0,007$ für Mutation m4 (Ayn98)) und einer japanischen Studie ($p<0,0001$ für Mutation m1 (Nak91)). Die Mutationen m2 und m1 lagen gekoppelt vor („linkage“) und bildeten das Allel *CYP1A1*2B*. Die Mutation m3, die bisher nur bei Afrikaner gefunden wurde, konnten wir nicht nachweisen. Die Häufigkeit der

CYP1A1 Allele mit hoher Aktivität, *CYP1A1*2A* und *CYP1A1*2B*, betrug 4,6% (3,1%-6,5%) bzw. 5,1% (3,5%-7,1%).

5,9% (3,5%-9,2%) aller Probanden waren CYP2D6 Langsam-Metabolisierer und 3,4% (1,7%-6,3%) wurden als Ultraschnell-Metabolisierer identifiziert (*CYP2D6*1x1/*1*). Das Mutationsspektrum von *CYP2D6* und das Verhältnis von intermediären und schnellen Metabolisierer in der russischen Stichprobe zeigten Gemeinsamkeiten mit anderen Studien an Kaukasiern.

Bei der Genotypisierung von acht verschiedenen Punktmutationen im *NAT2*-Gen ergab sich für 59,7% (54,1%-65,1%) der Studienteilnehmer ein Genotyp, der mit einer Langsam-Acetylierer-Status einhergeht. 34,7% (29,6%-40,2%) der Probanden hatten ein und 5,6% (3,3%-8,6%) zwei für die Schnellacetylierung kodierende Allele. Die Häufigkeitsverteilung der Punktmutationen, Allele und Genotypen des *NAT2*-Gens in diesem russischen Kollektiv zeigte viele Übereinstimmungen mit anderen kaukasischen Bevölkerungsgruppen. Wie bei anderen Europäern überwiegen auch in der russischen Bevölkerung aufgrund der Häufigkeit des Alleles **5* die Langsam-Acetylierer. Die für Afrikaner spezifische Variante 191G>A wurde nicht gefunden.

Die auf fluoreszenzmarkierten Sonden basierende Methode zur Genotypisierung der sechs Polymorphismen des *NAT2*-Gens mittels LightCyclers stimmte mit der konventionellen Bestimmung per PCR-RFLP in allen untersuchten Proben überein. Damit wurde das LightCycler-Verfahren als zuverlässige, schnelle und einfache Methode zur *NAT2*-Genotypisierung validiert.

Die Allelverteilung der für die wichtigsten Enzyme im Arzneimittelstoffwechsel kodierenden Gene ist bei Russen ähnlich wie bei anderen Kaukasiern. Es wurden weder asiatischer Einfluss gefunden noch die für Afrikaner charakteristischen Besonderheiten. Es kann deshalb erwartet werden, dass die genetisch-bedingten Unterschiede in der Wirksamkeit und im Auftreten von Arzneimittelnebenwirkungen in der russischen Bevölkerung vergleichbar sind mit denen in anderen europäischen Populationen.

Die von uns gesammelten populationsgenetischen Daten stellen eine wichtige Grundlage für weitere klinische Studien in der russischen Bevölkerung zum Einfluss genetischer Varianz auf die Arzneimittelverträglichkeit und die Rolle polymorpher arzneistoffmetabolisierenden Enzyme in der Pathogenese verschiedener Krebserkrankungen dar.

Die Genotypisierung von Enzympolymorphismen ermöglicht es, die Arzneimitteldosierung auf der Grundlage pharmakogenetischer Daten individuell anzupassen und damit das Risiko für unerwünschte Arzneimittelwirkungen oder Therapieversagen zu reduzieren.

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Eidesstattliche Erklärung

Hiermit erkläre ich an Eides statt, dass die vorliegende Dissertation von mir selbst und ohne die unzulässige Hilfe Dritter verfasst wurde, auch in Teilen keine Kopie anderer Arbeiten darstellt und die benutzten Hilfsmittel sowie die Literatur vollständig angegeben sind.

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